Glucocorticoid receptor effects on the immune system and inflammation.

Cover: The child and the immune cells have been subject of the studies described in this thesis. The blowing child symbolizes the cortisol effect on the direction of the immune cell response. The cells are human red and white blood cells, neutrophils and a mono-nuclear cell.

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Glucocorticoid Receptor Effects on the Immune System and Inflammation.

Glucocorticoid Receptor Effecten op het Immuum Systeem en Inflammatie.

PROEFSCHRIFT

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op gezag van de

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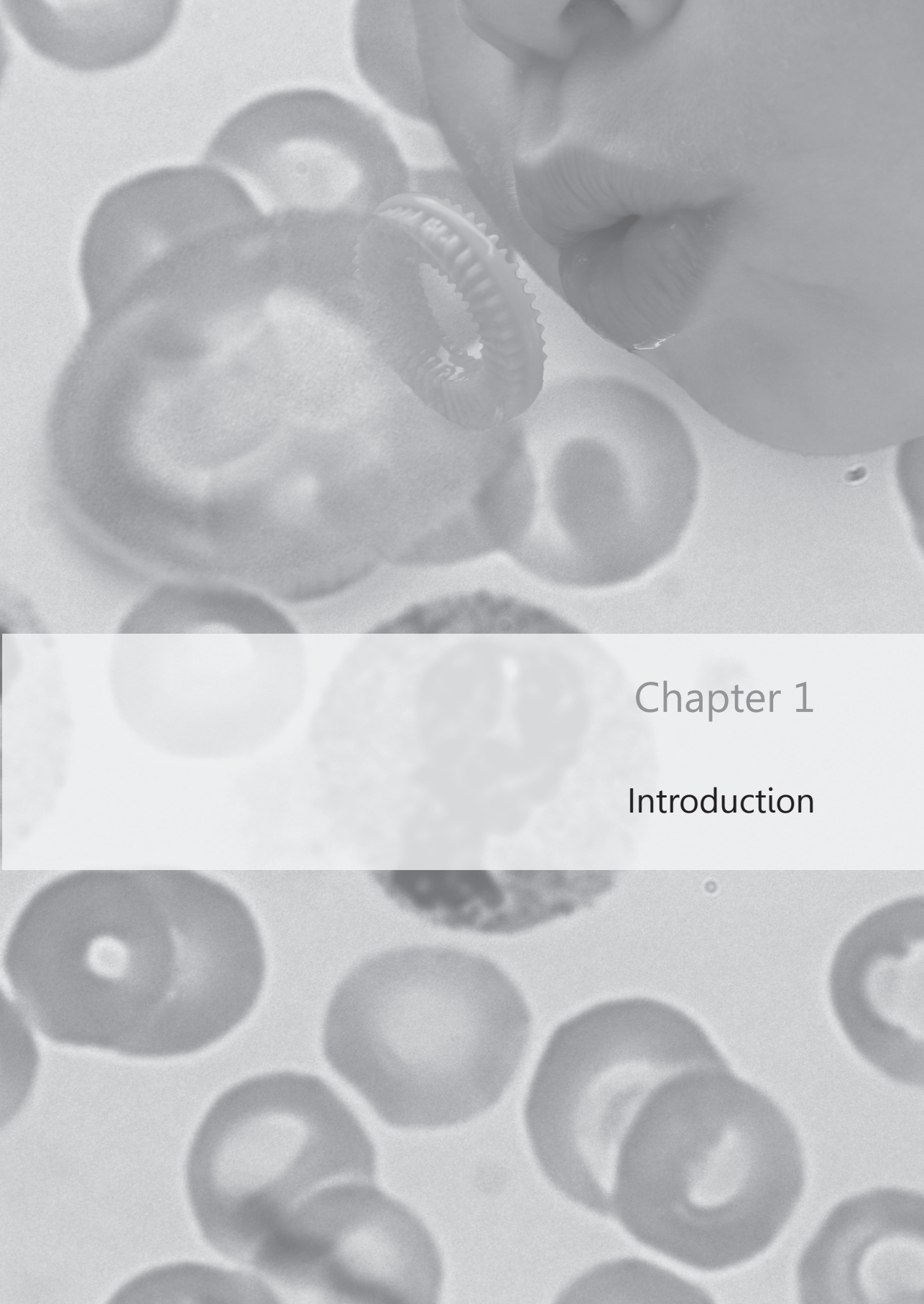
CONTENTS



Chapter 1

Introduction

INTRODUCTION

1.1 Glucocorticoids

Background
Biosynthesis and hypothalamo- pituitary-adrenal axis
Glucocorticoid actions - outside the immune system
- on the innate immune system
- on the acquired immune system

1.2 Glucocorticoid receptor

Gene structure
Alternative splicing
Posttranslational modifications

1.3 Glucocorticoid sensitivity

Glucocorticoid receptor gene polymorphisms
Measurement of glucocorticoid sensitivity in vivo: 0.25mg dexamethasone suppression test
Measurement of glucocorticoid sensitivity ex vivo: Glucocorticoid Induced Leucine Zipper / Interleukin-2 bioassay
Cortisol sensitivity disorders

1.4 Glucocorticoid receptor and sepsis

1.5 Aims of this thesis

References

1.1 GLUCOCORTICOIDS

Background

Thomas Addison's discovery in the mid-1800s that the adrenal cortex was essential for survival preceded by nearly a century the demonstration that this gland produced at least two distinct hormones, each essential for normal life. How glucocorticoids sustained life remained a mystery for decades. In 1949 glucocorticoids were found to have powerful anti-inflammatory activity, a discovery which led to their use as "miracle drugs" in many diseases [1]. Since their introduction in 1948, they have become so important that some authors divide the history into BC and AC (before cortisol and after cortisol) [2].
Today, glucocorticoids are among the most important drugs used in routine clinical practice because of their clinically important anti-inflammatory and immunosuppressive effects. The list of indications for glucocorticoid treatment is long. Diseases in which glucocorticoid treatment plays a major role include common disorders such as asthma, nephrotic syndrome, rheumatoid arthritis, dermatological diseases, Crohn's disease, systemic diseases, organ transplantation, and malignancies. However, serious adverse effects often accompany treatment. Many adverse effects are partly or mainly caused by glucocorticoid receptor transactivating effects. By contrast, anti-inflammatory effects are mostly mediated by glucocorticoid receptor transrepression. The challenge is balancing desired therapeutic effects and adverse reactions. Recently, population based individual variability in cortisol sensitivity and its implications for health profiles and risk for disease is coming into focus.

Biosynthesis and hypothalamo-pituitary-adrenal axis.

In humans the adrenal glands are triangle-shaped endocrine glands that sit on top of the kidneys; (ad= near + renes = kidneys). The adrenal cortex produces steroid hormones and is composed of three zones. The zona glomerulosa is mainly controlled by the renin-angiotensin system, which regulates the release of aldosterone which modulates both sodium and potassium homeostasis. The zona fasciculata is controlled by corticotrophin-releasing hormone (CRH) and corticotrophin (ACTH) system, which regulates the responses to stress through the actions of cortisol. The zona reticularis produces adrenal androgens. The adrenal medulla, located in the central part of the gland, is part of the sympathetic nervous system and produces catecholamines [1].
The hypothalamic-pituitary-adrenal (HPA) axis regulates cortisol secretion by a negative feedback system (Fig 1). Cortisol is secreted in response to ACTH, which is synthesized and released from the pituitary. ACTH is derived from the precursor pro-opiomelanocortin (POMC) and is secreted under the control of CRH and arginine vasopressin (AVP),

produced by the hypothalamus. ACTH binds to a G-protein coupled ACTH receptor in the membrane of cells in the zona fasciculata of the adrenal gland, leading to the activation of enzyme systems involved in the biosynthesis of steroids (Fig 2). Cortisol exerts a negative feedback on the pituitary and hypothalamus, reducing the output of ACTH and CRH, in order to reduce cortisol secretion. In this way, a balance between need and production is achieved [3].

In addition, the HPA-axis is controlled by the circadian rhythm and stressful events [4]. In healthy individuals, cortisol levels rise steadily after 4 a.m., peaking rapidly within 30-45 minutes after wakening. They then gradually fall during the day, rising again at night. This pattern is not present at birth. Estimates of when this pattern starts vary from two weeks to 9 months after birth [5]. There is also significant individual variation, although an individual tends to have consistent rhythms. Stress from diverse sources like fear, pain,

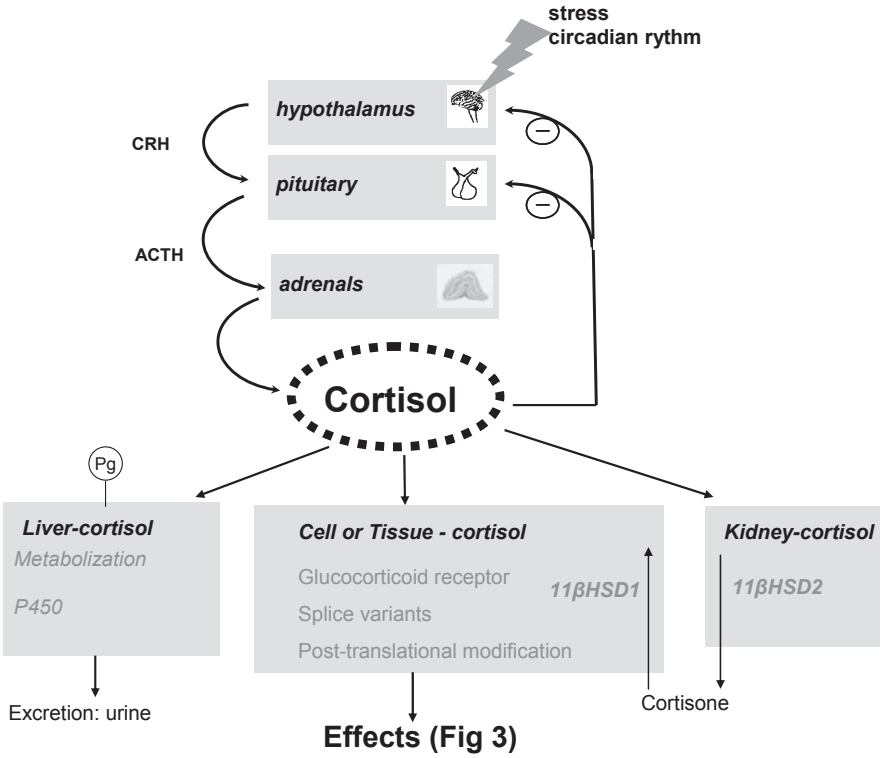


Figure 1. The hypothalamic-pituitary-adrenal axis. A simplified overview of the regulation of glucocorticoids and their effect. Under the influence of the circadian rhythm and stress (physiological or psychological), the hypothalamus secretes corticotrophin-releasing hormone (CRH) into the hypophysal portal system and thereby stimulates the production of adrenocorticotropic hormone (ACTH) by the pituitary. In response to increased levels of ACTH the adrenal glands increase the secretion of cortisol. Cortisol inhibits its own production both at the hypothalamic and pituitary level and thereby completing a negative feedback loop.

trauma, hemorrhage, cold, infection, hypoglycemia, emotional distress, inflammatory agents, heavy exercise and other challenges to homeostasis, stimulates the HPA-axis towards increased secretion of cortisol.

Most serum cortisol, 95% is bound to proteins including corticosteroid binding globulin (CBG), and serum albumin. Only free cortisol is available to the glucocorticoid receptors. Cortisol availablitily is also regulated at the tissue level by the 11-β hydroxysteroid dehydrogenase system (11-β HSD), which consists of two enzymes: 11-β HSD1 and 11-β HSD2 (Fig 1). 11-β HSD1 utilizes the cofactor NADPH to convert biologically inert cortisone to biologically active cortisol. 11-β HSD2 utilizes the cofactor NAD+ to convert cortisol to cortisone. Overall the net effect is that 11-β HSD1 serves to increase the intracellular concentrations of biologically active cortisol in a given tissue (such as the liver), while 11-β HSD2 serves to decrease the intra-cellular concentrations of biologically active cortisol especially in mineralocorticoid target tissues such as the kidneys, salivary glands and certain parts of the intestine.

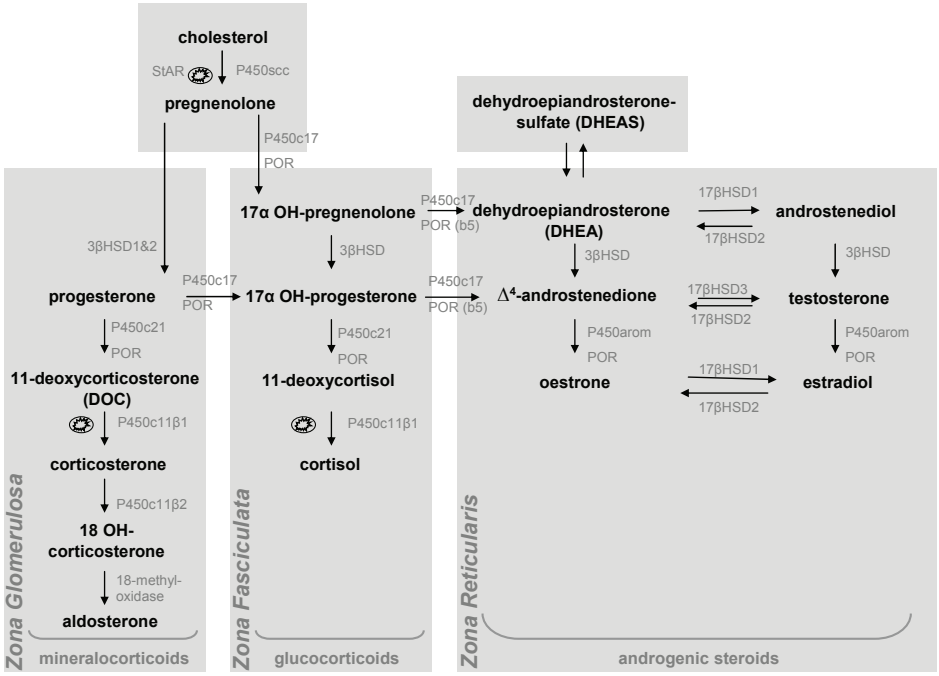


Figure 2. Biosynthesis of steroids in the adrenal cortex. Conversions occur in the microsomal compartment except for conversions marked with , which occur in mitochondria. StAR= steroidogenic acute regulatory protein, P450scc= cholesterol side chain cleavage enzyme. P450c17= 17α-hydroxylase or 17,20-lyase, P450c21= 21α-hydroxylase, P450c11β= 11β-hydroxylase, POR= P450 oxidoreductase, HSD= hydroxysteroid dehydrogenase, arom= aromatase.

Glucocorticoid actions outside the immune system

Most, if not all, cells in the body have glucocorticoid receptors, meaning they are sensitive to glucocorticoids. This leads to a diversity of physiological effects (Fig 3). Glucocorticoids act as antagonists to insulin by promoting gluconeogenesis, breakdown of lipids (lipolysis) and proteins, and mobilization of extra-hepatic amino acids and ketone bodies. This leads to increased blood glucose concentrations, resulting in increased glycogen formation in the liver. Glucocorticoids are crucial initiators of human osteoblast differentiation and matrix mineralization [6]. Glucocorticoids increase blood pressure, and increase the effectiveness of catecholamines, increase renal tubular acid secretion, probably through increased activity of the Na/H exchanger in the proximal tubule, and increase phosphaturia. They increase glomerular filtration rate (GFR), produce kaliuresis and natriuresis, and decrease synthesis of vasopressin [7]. Glucocorticoids influence behavior and mood and facilitate memory consolidation in physiological doses.
When given in supraphysiological doses like in corticosteroid therapies, glucocorticoids can lead to insulin resistance, diabetes mellitus, hypertension, and redistribution of body fat with and increase of visceral fat. They negatively influence bone formation resulting in osteoporosis and increased fracture risk [8]. Long-term exposure to glucocorticoids results in damage to cells in the hippocampus. This damage results in impaired learning[9].

Glucocorticoid actions on the immune and inflammatory system (Table 1).

Cortisol has an important role in balancing the immune response to infection. The general cortisol status of individuals has been suggested as an important determinant of susceptibility to infection. However, the physiological effects of cortisol on the immune system are poorly understood and many of the effects have been evaluated in conditions with supraphysiological concentrations of exogenously administered glucocorticoids. An adequate cortisol stress response of the HPA-axis is essential for surviving serious infections like sepsis. Circulating mediators of inflammation have a major role in activating the HPA-axis. Cytokines like TNF-alpha, interleukin-1 and interleukin-6, account for most of the HPA-axis stimulating activity during inflammation. Cortisol has an inhibitory effect on both innate and acquired immune function [10-12].

Glucocorticoid actions on the innate immune system.

GC act through protein-protein and protein-DNA interactions ultimately activating or repressing target gene transcription. Also non-genomic effects have been suggested [13]. Inhibition of chemotaxis and bactericidal activity in neutrophils and monocytes, lymphopenia, decreased macrophage function and disturbed complement function are well

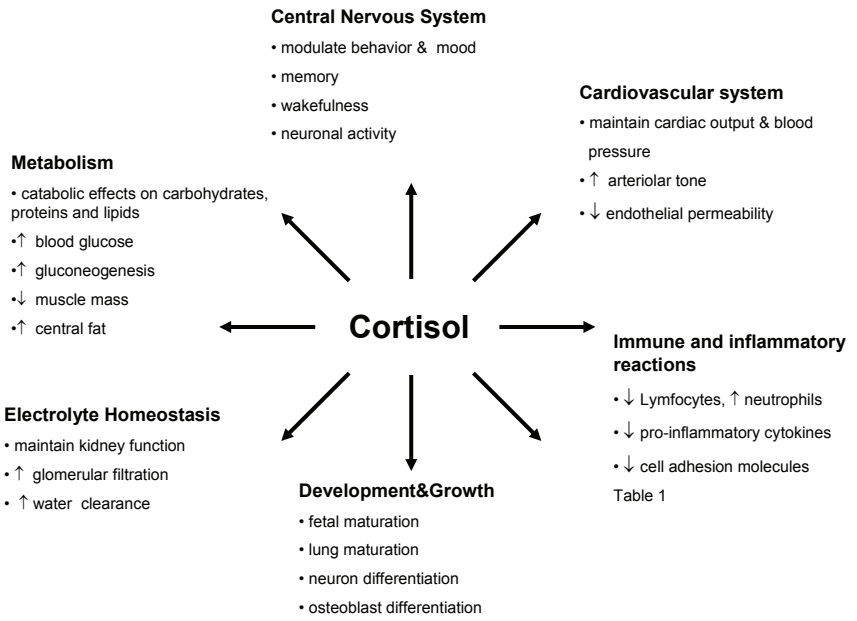


Figure 3. Overview of physiological cortisol effects.

known effects of GC administration [14]. Administration of corticosteroids has profound effects on the cellular functions of phagocytic cells and endothelial cells, resulting in reduced trafficking of leukocytes. Expression of adhesion molecules on the surfaces of both endothelial cells and leukocytes is markedly attenuated, resulting in a reduced accumulation of phagocytic cells at sites of inflammation. The reduction in endothelial adhesion function may be due to direct effects of glucocorticoids on expression of adhesion molecules as well as indirect effects due to the inhibitory effects of steroids on transcription of cytokines such as IL-1 or TNF that upregulate endothelial adhesion molecule expression. In addition to inhibiting transmigration of leukocytes, glucocorticoids may also attenuate the generation of inflammatory exudates by: 1) Upregulating the synthesis of angiotensin converting enzyme and neutral endopeptidase, enzymes that degrade bradykinin. 2) Suppressing production of inflammatory eicosanoids and arachidonic acid in phagocytic cells. 3) Suppressing the synthesis of cyclooxygenase-2 (COX-2), the inducible isoform of cyclooxygenase primarily responsible for production of prostaglandins at sites of tissue injury and inflammation.

The effects of GC primarily result from GR suppression of NF-kB transcription, a key player in inflammation, and via a crosstalk between GR and Toll-like receptors (TLR). TLR are responsible for the recognition of a variety of microbial pathogens and the initial induction of immune and inflammatory responses [15]. The effects of glucocorticoids on

Table 1 Effects of glucocorticoids on the immune system and inflammation

Leukocytes	Cytokine genes	Inflammatory agents	Cell adhesion molecules	Transcription factors
T- lymphocytes				
↓(Th2>Th1)	IL-1 ↓	Eicosanoids ↓	ICAM-1 ↓	NF-κB ↓
B-lymphocytes ↓/=	IL-2 ↓	Bradykinin ↓	LFA-1 ↓	AP-1 ↓
Neutrophils ↑	IL-3 ↓	5-HT ↓	E-Selectin ↓	NFAT ↓
Eosinophils ↓	IL-6 ↓	Histamine ↓	ELAM-1 ↓	STAT-6 ↓
Macrophages ↓	IL-8 ↓	Plasminogen activator ↓		GATA-3 ↓
Vascular migration ↓	IL-11 ↓	Collagenase ↓		T-bet ↓
Antibody production				
↑/=/↓	IL-12 ↓	Elastase ↓		TLR ↓
	IL-16 ↓	Cox-2 ↓		
	GM-CSF ↓	MMP-9 ↓		
	TNF-α ↓	iNOS ↓		
	IFN- γ ↓	Arachidonic acid ↓		
	IL-13 ↓	Monocyte chemoattractant protein-1 ↓		
	IL-5 ↓			
	IL-4 ↑	Lipocortin-1 ↑		IkBa ↑
	IL-10 ↑	α-2-macroglobulin ↑		IL-1RII ↑
	TGF-β ↑	Secretory leukocyte protease inhibitor ↑		

IL=Interleukin, GM-CSF= granulocyte macrophage colony stimulating factor, TNF= tumor necrosis factor, IFN= interferon, TGF= tissue growth factor, iNOS= nitric oxide synthase, NF= nuclear factor, STAT= signal transducers and activators of transcription, ICAM=intercellular adhesion molecule, NFAT= nuclear factor of activated T cells, TLR=Toll-like receptors white= pro-inflammatory agents and gray= anti-inflammatory agents

trafficking, survival, and effector functions of immune cells are are variable in different leukocyte cell types:

Neutrophils. Glucocorticoids markedly reduce the expression of adhesion molecules on the surfaces of both endothelial cells and leukocytes, resulting in a reduced accumulation of phagocytic cells at sites of inflammation. In contrast, glucocorticoids do not appear to significantly affect neutrophil phagocytic responses or bactericidal activity. Neutrophils of healthy persons are known for their low glucocorticoid receptor expression [16] and their reduced cortisol sensitivity compared to other immune cells [17].

Although trafficking to sites of inflammation is impaired, glucocorticoid treatment results in increased numbers of circulating neutrophils due to enhanced release of cells from the bone marrow, reduced vascular migration, and inhibition of neutrophil apoptosis.

Eosinophils. Treatment with glucocorticoids markedly decreases circulating levels of eosinophils. This is mediated in part by sequestration of eosinophils in extravascular tissues, an effect that may be due to the preferential upregulation of the chemokine receptor 4 (CXCR4). In contrast to their inhibitory effect on neutrophil apoptosis, glucocorticoids promote eosinophil apoptosis either directly or by attenuating synthesis of IL-5,

a cytokine that promotes eosinophil survival [18]. Glucocorticoids have variable inhibitory effects on the degranulation of eosinophils that are dependent upon the activating ligand and the corticosteroid used in the assay [19].

Macrophages. Corticosteroids decrease tissue accumulation of monocytes and macrophages, an effect mediated by decreased vascular migration and inhibition in the elaboration of macrophage migration inhibition factor. Antigen presentation and expression of class II-HLA molecules by macrophages is down regulated in response to glucocorticoids. In addition to diminishing the production of monocyte/macrophage derived eicosanoids and inflammatory cytokines (IL-1, TNF), glucocorticoids also inhibit macrophage phagocytic and microbicidal function [20].

Glucocorticoid actions on the acquired immune system

T cells. Corticosteroids play a physiologic role in the positive and negative selection of thymocytes with consequences for the development of the T cell repertoire. While glucocorticoids inhibit the acute generation of both Th1 and Th2 derived cytokines by activated T cells, the inhibitory effect on expression of Th1 cytokines appears to be greater [21].

B cells. Numbers of circulating B-lymphocytes are less affected by corticosteroid administration than are T cells. Acutely, corticosteroid treatment may promote B cell immunoglobulin secretion indirectly via inhibition of the CD8+ suppressor T cell function. Over a period of years, however, there may be a decrease in various antibody titers, possibly as a consequence of inhibition of T cell help and/or increased catabolism of generated antibody [22].

The inhibition of specific immune responses mediated by T cells and B cells is achieved by blocking the function of the transcription factors nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1) that are required for transcription of proinflammatory mediators [10]. This may be mediated in part by glucocorticoid-induced expression of MAPK phosphatase 1 and consequently dephosphorylation of various proteins that participate in intracellular signaling. Steroids increase the synthesis of IkappaB alpha (IκBα), a protein that traps and thereby inactivates NF-kB. In addition to their effects on gene transcription, glucocorticoids also inhibit secretion of inflammatory cytokines by affecting post-translational events. The stability of mRNAs encoding IL-1, IL-2, IL-6, IL-8, tumor necrosis factor (TNF), granulocyte-macrophage-colony stimulating factor (GM-CSF) and C-reactive protein are diminished in the presence of glucocorticoids [10-12].

1.2. THE GLUCOCORTICOID RECEPTOR

The effects of cortisol are exerted through the glucocorticoid receptor, although there is evidence that they can also act via a nongenomic pathway. The glucocorticoid receptor is found in cells throughout the body, including the brain. The presence of GR is essential for life and its absence in knock-out mice leads to death [23]. In humans, complete absence of the receptor is unknown.

Glucocorticoids, such as cortisol or its synthetic analogs, enter the cell by passive diffusion and bind to the GR (Fig 4). Upon glucocorticoid binding, the GR dissociates from an Hsp90 complex and translocates to the nucleus. Three mechanisms of action are known: 1) GR can activate or repress gene expression by interacting with specific DNA sequences, the hormone responsive elements, which are present in the promoter regions of steroid-responsive genes. The target genes that can be modulated by steroids vary

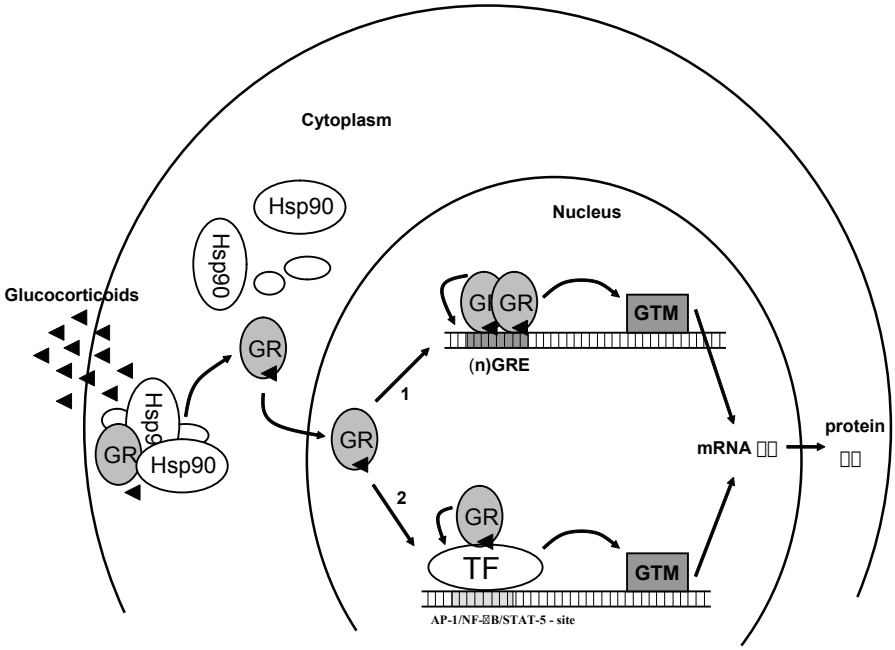


Figure 4. Simplified model of glucocorticoid receptor mediated transcriptional modulation. Glucocorticoids enter the cell by passive diffusion. Upon glucocorticoid binding, the receptor dissociates from a heat shock protein 90 (Hsp90) containing multi-protein complex and translocates to the nucleus. Once there, GR modulates target gene transcription via (1) direct interaction with (negative) glucocorticoid responsive elements (GRE) as a dimer and (2) cross-talk with other DNA bound transcription factors such as AP-1 and NF-κB. The resulting modulation of target gene transcription leads to altered protein expression. GRM= general transcription machinery; TF= other transcription factors. Reprinted with permission from thesis H.Russcher 2006.

greatly during development and also between tissues. 2) GR can interact with other transcription factors like AP-1 or NFkB, thereby repressing their transcriptional activity. NF-kB is one of the key pro-inflammatory regulators. It is activated by cytokines, microbial pathogens, viral infections and other triggering signals. Direct interaction between the GR and nuclear factor kappa B (NF-κB) inhibits NF-κB signalling [12]. 3) non-genomic activation. GCs can have rapid effects on inflammation that are not mediated by changes in gene expression. The non-genomic mechanism involves the activation of endothelial nitric oxide synthetase (eNOS). Activation of eNOS is stimulated in a GR-dependent, transcription-independent manner in human endothelial cells. This results in the production of nitric oxide, which is generally associated with vasodilation, migration of leukocytes across endothelium and inflammation [24].

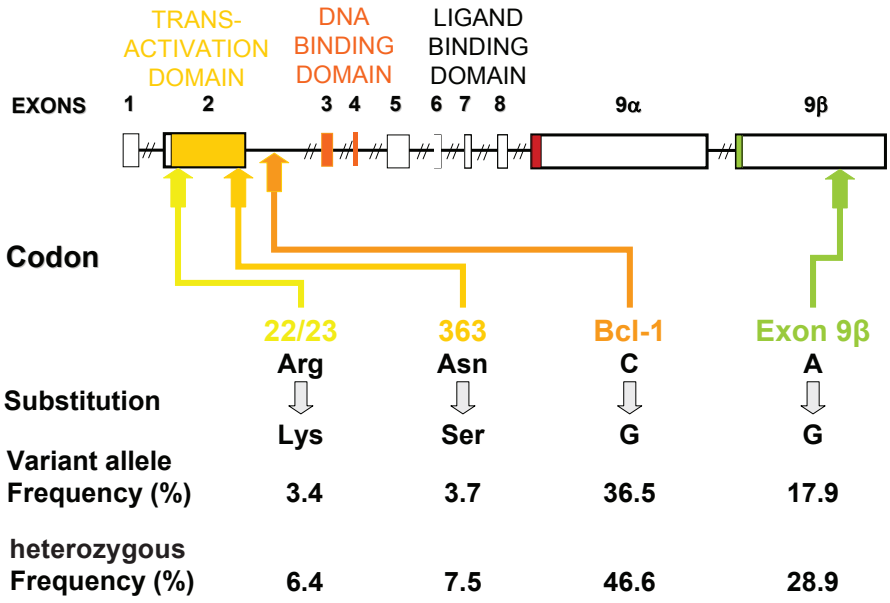


Figure 5. Schematic overview of the glucocorticoid receptor gene and its domains and four polymorphisms.

Gene structure

The GR gene (NR3C1) is located on chromosome 5. The GR belongs to the superfamily of nuclear receptors. All members of this superfamily share a characteristic three-domain structure: a) an amino-terminal transactivating domain (TAD), which directs transactivation of target genes, b) a DNA-binding domain, interacting with glucocorticoid response

elements (GRE) in the DNA and c) a carboxy-terminal ligand-binding domain, which contains specific steroid and heat shock protein binding sites [25-27]. The GR gene consists of 9 different exons (Fig 5).

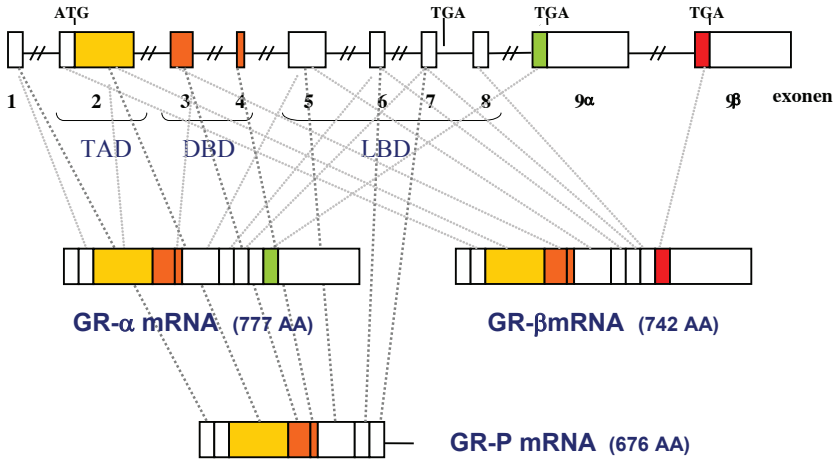


Figure 6. Schematic overview of the glucocorticoid receptor gene and alternative 3' mRNA transcripts. Alternative splicing of exon 9 gives rise to two mRNAs coding for GRα and GRβ; GR-P lacks exons 8 and 9.

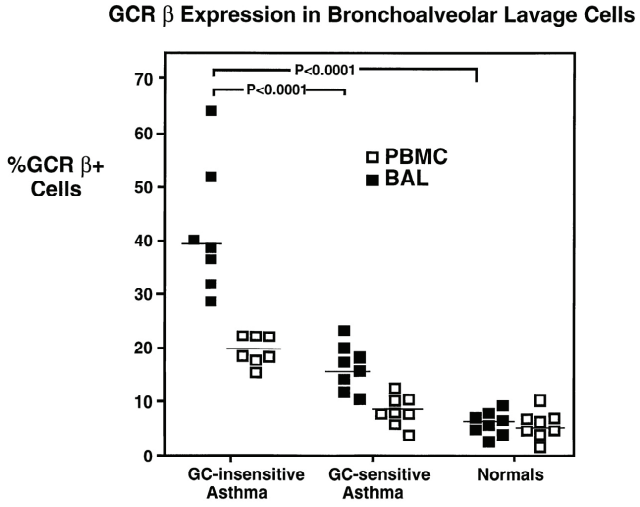


Figure 7. Increased glucocorticoid receptor beta expression has been found in airway cells of steroid resistant asthma patients. Reprinted from: Q.A. Hamid et al., Am J Respir Crit Care Med 159, 1600-1604, 1999.

Alternative splicing

Several alternative exons 1 were identified and designated as exon 1A, 1B and 1C, which are each preceded by their own promoter [28, 29].
Three different 3'-splice variants of the glucocorticoid receptor have been reported: GR-α, which binds ligand and is functionally active; GR-P, which is thought to activate the function of GR-α; and GR-β, which is a dominant negative inhibitor of GR-α action (Fig 6) [25, 29].
Abnormal levels of GR-α, GR-β and GR-P have been found in GC-resistant patients, mainly in diseases with a strong autoimmune component[30-33]. Increased GR-β expression was found in airway cells of glucocorticoid insensitive asthma patients (Fig 7) [31], in Hela cells treated with TNFα [34, 35] and in neutrophils after treatment with IL-8 [35] . Further, regulation of the different splice variants is thought to be involved in regulation of tissue specific glucocorticoid sensitivity [16, 36].

Posttranslational modifications.

The transcriptional activity of GR protein can be modulated by various posttranslational modifications, such as phosphorylation, ubiquitination, sumoylation and nitrosylation. These modifications result in alterations of protein-protein interactions, nuclear receptor DNA binding, binding to subcellular structures and degradation [29].

1.3. GLUCOCORTICOID SENSITIVITY IN HUMANS

Glucocorticoid receptor gene polymorphisms.

A large number of polymorphisms in the GR gene are known and the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.gov/SNP) currently lists 554 in humans. Few of them are associated with functionality (Fig 5). The TthIII 1 RFLP (rs10052957) [37], *ER22/23EK* (rs6189 and rs6190), *N363S* (rs6195), *Bcl1* (41423247) and *GR-9*β (rs6198) have been associated with changes in glucocorticoid sensitivity [38-40]. Of three polymorphisms, located in or near the transactivating domain, the associations with glucocorticoid sensitivity have been described extensively (Fig 8) [38]. The *ER22/23EK* polymorphism is associated with a relative resistance to GCs. This polymorphism influences translation by altering the secondary structure of GR mRNA, forcing translation of the mRNA into protein from codon AUG-1(GR-A) at the expense of initiation from codon AUG-27 (GR-B). The relative GC resistance is caused by the fact that the transactivating capacity of GR-A is lower than that of GR-B. Transrepression seems to be unchanged

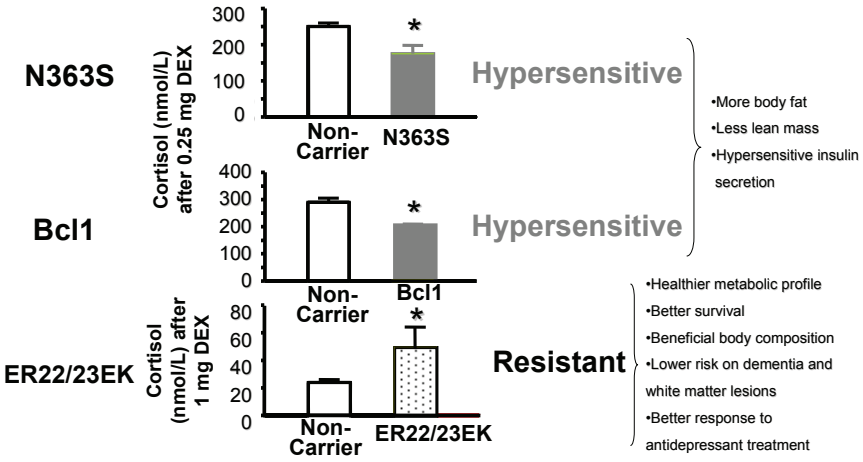


Figure 8. Three functional polymorphisms with a summary of their clinical associations. The graphs represent cortisol levels after dexamethasone (DEX) suppression tests. Non-carriers were compared to carriers of the polymorphism. Lower post-DEX cortisol levels (N363S and Bcl1) suggest a hypersensitivity to glucocorticoids. Higher post-DEX cortisol levels (ER22/23EK) suggest a relative resistance to glucocorticoids. van Rossum & Lamberts, Rec Prog Horm Res, 2004

because the GR-A and GR-B isoforms are equally potent at inhibiting the transactivating activity of NF-kB [41, 42]. The 22/23EK carriers showed a reduced response (had higher cortisol levels) after dexamethasone suppression testing. Their metabolic profile showed lower total cholesterol and low-density lipoprotein cholesterol levels, lower fasting insulin concentrations, an increased insulin sensitivity [43] as well as lower C-reactive protein (CRP) levels. The *N363S* and *Bcl1* polymorphisms have been associated with increased GC sensitivity, decreased insulin sensitivity to dexamethasone and increased abdominal obesity. The molecular mechanism of their effects remains to be elucidated. The fourth polymorphism, *GR-9*β, is an A to G nucleotide substitution located in the 3' UTR of exon 9β, the terminal exon of the mRNA of the β isoform (nucleotide 3669 in X03348; rs 6198). The A to G nucleotide substitution is located in an 'ATTTA' motif (changing it to GTTTA). This 'ATTTA' motif is known to destabilize mRNA and decrease receptor protein expression in vitro [39]. The β isoform has been reported to have a dominant negative effect on GRα action [25, 44]. In vitro data show that the *GR-9*β polymorphism leads to a more stable GRβ mRNA and possibly to a relative GC resistance and increased susceptibility to rheumatoid arthritis [40]. However, in a low-dose dexamethasone suppression test, no differences in the cortisol levels after dexamethasone were found [45].

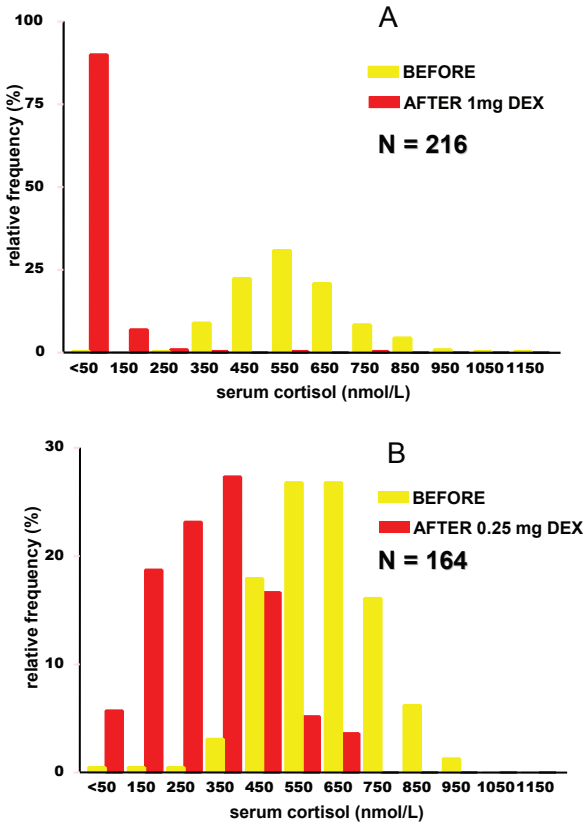


Figure 9. Cortisol concentrations before and after dexamethasone administration. The relative distribution of cortisol concentrations before (yellow bars) and after (red bars) 1 mg (a) or 0.25 mg (b) of dexamethasone (DEX) in healthy individuals. Blood samples were drawn between 8:00-9:00 a.m. after DEX was administered at 11:00 p.m. the previous night. Huizenga et al. JCEM, 1998

Measurement of glucocorticoid sensitivity in vivo: dexamethasone suppression test.

Sensitivity to endogenous and exogenous cortisol is highly variable between individuals. However intra-individual cortisol sensitivity is stable, suggesting genetic factors determining the sensitivity [46]. Basal concentrations of plasma cortisol exhibit a wide variation between normal subjects, but show an individual stability, indicating that the HPA axis is set at a stable and reproducible set point for a given individual.

The sensitivity of the HPA-axis feedback system to glucocorticoids can be measured by a dexamethasone suppression test (DST). The 1-mg overnight DST is used as a screening procedure for discriminating patients with Cushing disease from healthy individuals.

After administration of 1 mg of DEX at 2300 h to normal subjects, the nocturnal surge in ACTH production and, as a consequence, cortisol levels are normally suppressed < 145 nmol/L when measured the next morning (Fig 9a). It became clear from population based studies, that in the DST with 0.25-mg, subjects with the highest baseline cortisol concentrations also had the highest post-DEX cortisol concentrations.[46] Within an individual, there seems to be a set point for HPA activity, which is defined before as well as after a low dose of DEX. A dose of 1 mg DEX has too much suppressive effect to demonstrate this phenomenon, but a dose of 0.25 mg DEX, which results in a subtotal suppression of cortisol levels, leaves the influence of the individual's set point of the HPA axis intact. In the 0.25 mg DST, post-dex cortisol levels show a Gaussian distribution (Fig 9b), where the subjects in the extremes are relatively hypersensitive or resistant to glucocorticoids.

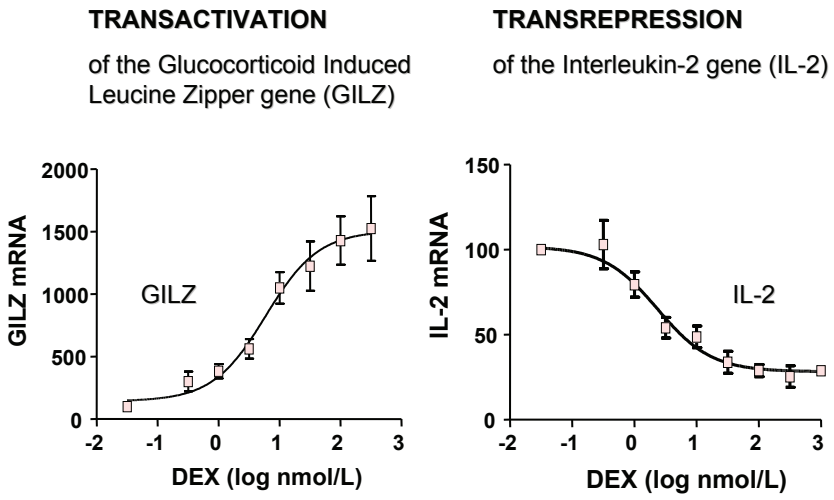


Figure 10. Relative responses of glucocorticoid-induced leucine zipper (GILZ) and Interleukin-2 mRNA expression in peripheral blood mononuclear cells of healthy individuals (n=10). Y-axis: control =100%. Russcher et al., JCEM 2005

Measurement of glucocorticoid sensitivity ex vivo: GILZ / IL-2 bioassay

There are only a few bioassays available that allow us to study variability in GC sensitivity. These are summarized in table 2 [47-51]. We recently developed an assay, which can directly measure GC- sensitivity by looking at GC-effects on gene expression, both by transactivation and by transrepression (Fig 10). The expression of the glucocorticoid induced leucine zipper (GILZ) gene is strongly stimulated by GCs [52]. Transactivation occurs via di-

Table 2: Bioassays to study variability in GC sensitivity

* DEX suppression assay (Maguire KP 1987)
The degree of DEX-induced suppression of endogenous cortisol levels via the strong HPA-axis feedback system is an index of an individual's GC sensitivity.

* Skin blanching assay (McKenzie 1992)
GCs on skin cause vasoconstriction. The resulting white spot should correlate with the GC sensitivity of the exposed individual.

* Proliferation assay (Lamberts 1992)
Mitogen stimulated proliferation of T-lymphocytes, which could be measured by incorporation of [3H]-thymidine, could be dose-dependently inhibited by DEX. Emax and EC50 values could serve as an index of an individual's GC sensitivity.

* Cytokine production assay (Ebrecht 2000)
The release of the proinflammatory cytokines IL-6 and TNFα from monocytes and macrophages, measurable by RIA's could be dose-dependently inhibited by DEX. Emax and EC50 values could serve as an index of an individual's GC sensitivity.

* FKBP51 mRNA expression assay (Vermeer H JCEM 2003)
The dose-dependently activated expression of FKBP51 mRNA by GCs could be quantified by real-time RT-PCR and the resulting Emax and EC50 values could serve as an index of an individual's GC sensitivity.

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rect interaction of the GR with the GC response elements (GREs) in the promoter region of the GILZ gene. The expression of the Interleukin-2 gene is strongly repressed by GCs. Transrepression occurs via direct protein interaction of GR and NFkB. Alterations in GILZ and IL-2 mRNA expression, in response to dexamethasone, are measured in peripheral blood mononuclear cells with a sensitive quantitative real-time PCR. The E_{max} values can serve as an index of an individual's GC sensitivity [48].

Cortisol sensitivity disorders

The pathologic extremes of variability in cortisol sensitivity can be divided in cortisol resistance and hypersensitivity. Until now, only few studies of these disorders have been described (Table 3) [53]. In cortisol hypersensitivity, the patient presents with the symptoms of Cushing's syndrome accompanied by hypocortisolemia [54]. Cortisol resistance (Fig 11) is characterized by hypercortisolism without cushingoid features (Fig 11) [55, 56]. The negative feedback on the HPA axis is reduced, due to diminished GC sensitivity, resulting in higher cortisol secretion by the adrenal glands to keep balance between need and production. However, adrenal production of mineralocorticoids is also increased, causing

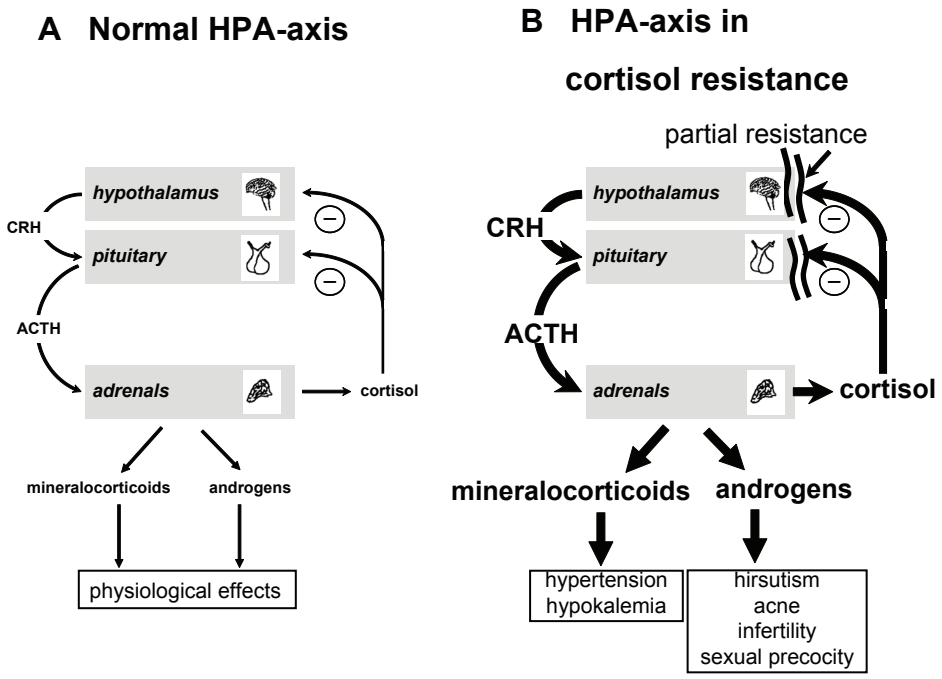

Figure 11. Schematic model of the pathophysiological mechanism in cortisol resistance. As a result of generalized glucocorticoid resistance, the HPA-axis is set at a higher level, resulting in an adrenal overproduction of glucocorticoids (cortisol), mineralocorticoids and androgens. The increased GC level compensates for the GC resistance and does not lead to clinical signs of hypercortisolism. The overproduction of mineralocorticoids and androgens lead to signs and symptoms of the clinical syndrome of cortisol resistance.

the symptoms of hypertension and hypokalemia. And the increased levels of adrenal androgens cause acne, hirsutism, virilisation, male pattern of baldness, menstrual irregularity and infertility. In children the presenting symptom can be premature adrenarche and precocious puberty. In a few kindreds the underlying molecular basis has been revealed--e.g. mutations in the gene coding for the GR--but in a substantial number of patients the cause of GC resistance has not yet been elucidated [57]. Diagnosis of generalized GC resistance can be difficult and will be further addressed in the discussion.

1.4 GLUCOCORTICOID RECEPTOR AND SEPSIS

Sepsis is a systemic response to a severe infectious disease with still a high mortality and morbidity despite improving treatments. Cortisol has an important role in balancing the immune response to infection. An adequate cortisol response to stress is essential

Table 3. Classification of Human Glucocorticoid Resistance Syndromes

Type of resistance	Underlying mechanism
I. Generalized inherited (familial) GC resistance (GIGR)	Mutations in GR gene
II. Pharmacologically induced GC resistance	
A. Administration of GR antagonist RU486	Blocks ligand binding to GR protein and activates HPA by preventing negative feedback mediated by GCs
B. Treatment of leukemic cell line with chemotherapeutic drugs	Deletion of GR gene
C. Treatment of leukemic cell line with chemical mutagens	Deletion of GR gene
III. Acquired GC resistance	
A. Neoplastic	
1. Ectopic ACTH syndrome	Decreased GR number, truncated GR, aberrant splicing and mutation of GR
2. Pituitary tumors (Nelson's syndrome)	Mutation of GR
3. Hematological malignancies	Mutations and aberrant splicing of GR
B. Transient	
1. Depression	Decreased number of GR
2. AIDS	Increased number of GR and reduced ligand affinity
3. Steroid resistant asthma, rheumatoid arthritis	Number of GR abnormalities reported
IV. Physiological resistance to GCs and variation in sensitivity	
A. Receptor downregulation	GCs decrease rate of transcription of GR gene and decreased sensitivity
B. Distal nephron of kidney	11β-Hydoxysteroid dehydrogenase catalyzes conversion of cortisol to cortisone, which is an inactive GC.
C. Individual differences in GC sensitivity	Genetic variation in control of cortisol secretion, regulation of HPA-axis, and GR expression

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for sepsis survival [58], but treatment with high doses of corticosteroids does not improve outcome. Low-dose corticosteroid replacement in adult patients with septic shock has a beneficial effect on outcome [59]. Discussion on which patient will benefit from corticosteroid treatment is still ongoing [60]. Inter-individual variations in the endogenous cortisol response to stress, mediated by the HPA-axis, might play a role in the explanation of the observed differences. The cortisol stress response has been studied in children with sepsis. Survivors showed higher cortisol levels compared to non-survivors [61, 62].
However, cortisol effect is regulated by the glucocorticoid receptor, at a tissue level. In lymphocytes of sepsis patients, increased cortisol sensitivity has been found compared to controls [63]. Other studies reported glucocorticoid receptor down-regulation as well as up-regulation in different tissues, measured by protein expression (western blots) or by binding-assays. In endotoxin treated rats, reduced GR binding in liver, lung and spleen was seen [64, 65]. However, increased GR expression and binding activity were found in muscle [66]. Previous in vitro studies showed that after stimulation of neutrophils with pro-inflammatory cytokines, a lower GR-α / GR-β ratio was found [29, 35, 67]. The currently available technologies provide the possibility of measuring mRNA expression quantitatively. Activated neutrophils are important in fighting sepsis and lead to an enhanced innate immune response to bacterial invasion [68]. Glucocorticoids support this process by increasing granulocyte number and activation status [69]. This could be a useful adaptation in the acute phase of sepsis when fighting the bacterial infection has priority. However, in case of prolonged sepsis, as is seen more often in adults compared to children, this change in neutrophil activation status might lead to tissue damage, multiple organ failure and thus does not benefit the patient. We hypothesized that regulation of neutrophil activity by changing its cortisol sensitivity, due to different splicing, during sepsis could therefore enhance pathogen elimination or diminish organ failure.

1.5 AIMS

The aims of this thesis were:
Characterization of genomic and post-genomic glucocorticoid receptor effects on cortisol sensitivity.

Chapter 2

To study the association of the glucocorticoid receptor haplotype characterized by the glucocorticoid receptor-*9*β polymorphism, with HPA-axis feedback, anthropometric data and lipids.

To study the effects of the glucocorticoid receptor haplotype, characterized by the glucocorticoid receptor-9β polymorphism, on GC transactivation and transrepression.

Chapter 3

To study the association of glucocorticoid receptor polymorphisms with parameters of immune system activity like microbial colonization.

Chapter 4

To study the association of glucocorticoid receptor polymorphisms with intermediates, like CRP, and end-point parameters of inflammation like atherosclerosis and cardiovascular disease.

Chapter 5

To develop strategies for characterization of cortisol sensitivity in patients with cortisol resistance or hypersensitivity.

Chapter 6+7

To study the endogenous post-genomic glucocorticoid receptor changes during sepsis longitudinally.

To study the change of glucocorticoid receptor splice variant levels in sepsis patients with different severity of illness, biochemical parameters of cortisol, inflammation and with use of medication in children with sepsis.

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Chapter 2

Glucocorticoid receptor polymorphism affects transrepression but not transactivation.

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ABSTRACT

Context:
Glucocorticoids (GCs) are extensively used in the treatment of inflammatory and autoimmune diseases. Their beneficial effects are thought to be mediated by GC transrepression on gene expression. However serious adverse effects, presumably mediated by GC transactivation of gene expression, limit their use.

Objective:
The effect of the glucocorticoid receptor haplotype, characterized by the *GR-9*β polymorphism, on GC transactivation and transrepression.

Design and methods:
A cross-sectional study in 216 persons randomly selected from participants in The Rotterdam Study, a population-based cohort study in the elderly. Clinical and biochemical parameters of GC sensitivity were measured: weight, height, waist-hip-ratio, glucose, insulin, total cholesterol, high-density lipoprotein, and C-reactive protein. In a dexamethasone suppression test the response of serum cortisol concentrations was studied. Genotyping for 4 GR polymorphisms was performed. In addition, ex vivo experiments were performed with leukocytes of 10 healthy controls and 2 persons homozygous for the *GR-9*β polymorphism, in which the expression of two GC-sensitive genes, GC induced leucine zipper (GILZ) and interleukin-2 (IL-2), was measured.

Results:
Persons carrying the *GR-9*β haplotype without 22/23EK (n=53) revealed no significant differences in their body mass index, waist-hip-ratio, fat spectrum, and insulin sensitivity, nor in their cortisol response to dexamethasone and levels of C-reactive protein, compared to non-carriers (n=113). Ex vivo, GC-induced upregulation of GILZ mRNA via transactivation did not significantly differ in *GR-9*β homozygotes, while the downregulation of IL-2 expression via transrepression was decreased.

Conclusion: Persons carrying the *GR-9*β haplotype seem to have a decreased GC transrepression with normal transactivation.

INTRODUCTION

Glucocorticoids (GC) are important regulators in a variety of processes such as body composition, metabolism, and immune function. GCs exert their effects via the glucocorticoid receptor (GR), a ligand dependent transcription factor, which belongs to the superfamily of nuclear receptors. GCs have both stimulatory and inhibitory effects on gene expression[1]. Their transrepressive effects are extensively used in the treatment of inflammatory and autoimmune diseases. However their practical use is limited by serious adverse effects, which are presumed to be mainly mediated by GC transactivation[2]. Four functional polymorphisms in the GR gene have been implicated in the inter-individual diversity of cortisol sensitivity[3, 4]. Of three polymorphisms, located in or near the transactivating domain, the associations with GC-sensitivity were described[5, 6]. The *ER22/23EK* polymorphism is associated with a relative resistance to GCs. The 22/23EK carriers had higher cortisol levels after dexamethasone suppression testing[5]. Their metabolic profile showed lower total- and low-density lipoprotein cholesterol-levels, lower fasting insulin concentrations, an increased insulin sensitivity[7] as well as lower C-reactive protein (CRP) levels[5]. The *N363S* and *Bcl1* polymorphisms have been associated with increased GC-sensitivity, decreased insulin sensitivity to dexamethasone and increased abdominal obesity[5, 8].

The fourth polymorphism, *GR-9*β, is an A to G nucleotide substitution located in the 3'-UTR of exon 9β, the terminal exon of the mRNA of the GRβ isoform (rs 6198). The A to G nucleotide substitution is located in an 'ATTTA' motif (to GTTTA). This 'ATTTA' motif is known to destabilize mRNA and decrease receptor protein expression in vitro[9]. The GRβ splice variant was reported as a dominant negative inhibitor of GRα action[10]. In vitro data show that the *GR-9*β polymorphism leads to a more stable GRβ mRNA and possibly to a relative GC-resistance. In addition, *GR-9*β was associated with decreased susceptibility to nasal colonization by Staphylococcus aureus[11] and increased susceptibility to rheumatoid arthritis[12], suggesting a GR-mediated effect on the immune system.

The aim of our study was to explore the possible role of the *GR-9*β polymorphism on several endpoints of GC sensitivity in a cohort of healthy elderly subjects.

MATERIALS & METHODS

Study population

This cross-sectional study was conducted as part of the Rotterdam Study, a prospective, population-based cohort study on determinants of disease and disability in elderly persons started in Rotterdam, The Netherlands in 1990 among 7983 participants[13]. A total

of 216 randomly selected persons participated in the present study. The Medical Ethics Committee of the Erasmus MC approved the study. Informed consent and permission to retrieve information from treating physicians was obtained from all participants. For the in vitro experiments, a group of 75 healthy volunteers was genotyped, resulting in the identification of 2 persons homozygous for the GR9β polymorphism and 10 controls homozygous for the reference allele.

Dexamethasone suppression test

The dexamethasone suppression test (DST) was performed as described previously[3]. Procedures for measurements of serum cortisol, insulin, DEX, glucose, total cholesterol, high-density lipoprotein (HDL), cholesterol and CRP were described previously[7]. For CRP analysis subjects with possible acute inflammation (CRP>10 mg/L) were excluded.

Genotyping

DNA was isolated from leukocytes using standard techniques. Genotyping was performed using 5 ng DNA in a Taqman allelic discrimination assay (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands). on the Taqman Prism7900HT (Applied Biosystems). All participants were genotyped for the *GR-9*β polymorphism. The genotypes for the *ER22/23EK*, *N363S* and *Bcl1* polymorphisms have been identified previously [7, 14, 15].

We used the genotype data for each of the 4 polymorphisms to infer the haplotypes present in the population using the program PHASE[16]. Three of the four polymorphisms were found to be mutually exclusive, only the codon 23 A-allele was always present in combination with the GR9β G-allele. Haplotype2, 3, 4 and 5 were characterized by carrying the Bcl, *GR-9*β, 363S and 22/23EK+GR-9β polymorphisms respectively. In haplotype 5, the 22/23EK polymorphism is always accompanied by *GR-9*β, but not vice versa (haplotype3).

GC induced leucine zipper (GILZ) and Interleukin-2 (IL-2) expression assay

The expression of GILZ and IL-2 mRNA in response to DEX was measured using real-time quantitative PCR as described previously [17, 18] in peripheral blood mononuclear leukocytes (PBMLs) of 10 non-carrier controls (5 men and 5 women, aged 25-38) and 2 homozygous *GR-9*β carriers (both men, aged 27 and 25). All were healthy volunteers without a history of GC medication. Briefly, PBMLs were isolated using standard techniques, pre-cultured for 24 h and subsequently incubated for 4 h with a range of DEX concentrations (0-1000 nmol/L) and 10 μg/mL phytohemagglutinin. Cells were subsequently washed with NaCl (0.15 mol/L), total RNA was isolated, and cDNA was synthesized in a reverse transcrip-

tion reaction. GILZ and IL-2 expression levels were normalized to the expression of the housekeeping gene hypoxanthine phospho-ribosyl transferase using the comparative treshold cycle (Ct) method[19].

Statistical analysis

Data were analyzed using SPSS for windows, release 10.1 (SPSS, Chicago, IL, USA). Logarithmic transformations were applied to normalize variables. Because of their linkage disequilibrium, we analyzed *GR-9*β while excluding *ER22/23EK* carriers. Differences in means of continuous variables between G-allele carriers and non-carriers of the *GR-9*β polymorphism were tested by ANCOVA using the general linear model procedure adjusted for age and sex. The Pearson Chi-square test was used for differences between categorized variables and genotypes. Bonferroni correction was used to correct for multiple testing. Differences in the EC_{50} to DEX-induced up- and down-regulation of GILZ and IL-2 mRNA levels in homozygous *GR-9*β carriers compared to non-carriers were analyzed by the Student's *t*-test using the area under the curve. Results are reported as means±SE. Two-sided *P* values of equal or less than 0.05 were considered to indicate statistical significance.

RESULTS

For 187 of the 216 participants the complete dataset, confounding variables and the GR genotype were available. Mean age was 67 years, 96 (51%) were women. We found a high allele frequency (16%) of 9β (haplotype3) while the 9β + *ER22/23EK* (haplotype 5) was less frequent (4,5%)[7].The distribution of genotypes for all GR gene polymorphisms was in Hardy-Weinberg equilibrium ($p>0.05$).

Comparison of means between non-carriers and the G-allele carriers of the *GR-9*β polymorphism (haplotype3) revealed no significant differences for cortisol response in the DST, nor in BMI, WHR, insulin sensitivity, cholesterol, LDL and HDL and hs-CRP levels after Bonferroni correction for multiple comparisons (Table 1).

In a previous report[18], we described the effects of haplotype4 and 5 on the regulation of two endogenous GC-sensitive genes: transactivation of the GILZ gene, and transrepression of the IL-2 gene. In haplotype4 and 5 increased and decreased transactivation were found, respectively, while transinhibition was not affected. In order to carry out similar experiments with respect to the *GR-9*β polymorphism, we identified 2 homozygous 9β carriers and 10 non-carriers among 75 volunteers. Figure 1 shows the reponse of these genes to DEX in PBMLs of homozygous carriers of the *GR-9*β polymorphism (haplotype3) compared to controls. Upregulation of GILZ mRNA did not significantly differ, while in

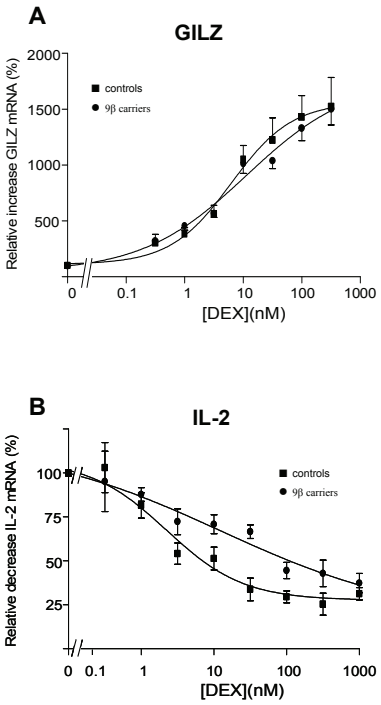

Figure 1: Relative responses to DEX of GILZ mRNA (A) and IL-2 mRNA (B) expression in PBMLs of noncarrier controls (haplotype1, n=10, squares) and homozygous 9β carriers (haplotype3, n=2, circles). PBMLs of homozygous carriers of the 9β polymorphism (n=2) and healthy non-carrier controls (n=10) were incubated during 4h with PHA and the indicated concentrations of DEX, followed by mRNA isolation and quantification by real-time Q-PCR. Data are presented as the increase of GILZ mRNA (A) and decrease of IL-2 mRNA (B) relative to the values in the absence of DEX. Duplicate assays at different time points were performed for every subject. Duplicate DEX incubations and duplicate Q-PCR was performed for every sample. PHA was necessary to induce transcription of IL-2, but did not affect GILZ or GR mRNA expression levels (not shown). No systemic differences before stimulation were observed between both haplotypes for both mRNAs (not shown)

the downregulation of IL-2 expression the mean EC_{50}, was shifted to the right for *GR-9*β carriers (13.0±1.4 nmol/L) compared to controls (5.2±1.3 nmol/L, $p<0.05$), indicating reduced GC sensitivity in their transrepression(Figure 1). Expression levels of the GRβ splice variant mRNA were approximately 1000-fold lower than those of the GRα mRNA, both in the GR9β carriers and in controls. Furthermore, we did not find higher GRβ splice variant expression levels in homozygous 9β carriers than in controls (data not shown).

TABLE 1. Genotypes of the study group in relation to the results of the 1 mg dexamethasone suppression test and metabolic parameters.

	non carriers	9beta carriers (haplotype3)	
	mean (se)	mean (se)	p
Gender = female	54 (48%)	31 (59%)	0.20
Age (years)	66.2 (0.6)	67.5 (0.8)	0.21
DST (1mg), n=	113	53	
fasting cortisol (nmol/L)	513 (13.6)	523 (17.3)	0.54
post dex cortisol (nmol/L)	26 (2.0)	27 (2.2)	0.7
fasting CBG (pmol/L)	737 (19.7)	764 (24.7)	0.21
free fasting cortisol (nmol/L)	37 (2.8)	30 (2.3)	0.31
free cortisol post dex (nmol/L)	1 (0.2)	0.8 (0.15)	0.85
fasting glucose (mmol/L)	5.8 (0.7)	5.9 (0.2)	0.58
fasting insulin (mU/L)	14 (0.7)	13 (1.1)	0.49
BMI (kg/m2)	26.6 (0.33)	25.8 (0.53)	0.18
WHR	0.93 (0.009)	0.90 (0.01)	0.61
total chol (mmol/L)	6.9 (0.11)	6.9 (0.17)	0.96
LDL (mmol/L)	5.1 (0.11)	5.2 (0.16)	0.9
HDL (mmol/L)	1.3 (0.04)	1.4 (0.05)	0.7
CRP (mg/L)	2.5 (0.21)	2.8 (0.31)	0.05[A]

DST = Dexamethasone suppression test, se=standard error.

P-values are corrected for age and sexe,. [A]: p corrected for age, sexe and BMI.

DISCUSSION

GR-9β, has been reported to result in stabilization of GRβ mRNA, which has dominant negative effects on GRα[9], resulting in a relative GC resistance leading to reduced immune suppression. This was supported by the higher frequency of this polymorphism found in rheumatoid arthritis patients[12] and the reduced risk for *Staphylococcus aureus* nasal carriage found in haplotype3 carriers[11]. In this study we did not find evidence for a reduced GC sensitivity in carriers of the *GR-9β* polymorphism compared to non-carriers, at the level of cortisol suppression during the DST, nor in BMI, WHR, insulin sensitivity, lipid profile and CRP.

Several other polymorphisms in the GR gene were found to be associated with glucocorticoid sensitivity. 22/23EK was shown to result in lower GR-mediated transcriptional activation, due to a shift in translation of the GR mRNA from GR-B to GR-A[6]. This reduced GC sensitivity is thought to be the cause of the decreased level of cortisol suppression during the DST, lower BMI, lower WHR and healthier lipid profile in 22/23EK carriers compared to non-carriers. Our study demonstrates that these previously described as-

sociations of *ER22/23EK* polymorphism with cortisol resistance, are not the result of its linkage disequilibrium with the 9β polymorphism.

The effects of the GR on HPA-activity, BMI and lipids are exerted via the transactivating pathway. For transactivation GR ligand binding, dimerization and DNA binding are needed, while GR effects on inflammation are presumed to be mainly mediated by direct protein-protein interactions with AP-1 or NFkB[20]. Earlier investigations offer some indications that these are indeed separate processes for different GR haplotypes. We previously reported ex vivo data, indicating that haplotype4 and 5 directly affected gene expression through transactivation, while transrepression seemed to be unchanged[18]. In the present study, we also studied this for haplotype3 (Figure 1). Transactivation of GILZ was not influenced by haplotype3 compared to controls, while transrepression of IL-2 was significantly reduced. The mean EC_{50} for IL-2 suppression was significantly higher in *GR-9*β homozygotes (13.0 ±1.4 nmol/L) than in controls (5.2 ±1.3 nmol/L, $p<0.05$). Thus haplotype3 showed a reduced effect in transcriptional repression and this could lead to less GR-mediated immune suppression. Although data from the literature remain controversial on the effect of the GRβ splice variant on direct protein-protein interactions (via NF-κB)[21, 22], our ex vivo data support the hypothesis that the *GR-9*β polymorphism and the GRβ splice variant may have an important role in human immune cells. The β splice variant expression is tissue specific and shows high expression in immune cells[23]. Therefore the *GR-9*β effects may be limited to tissues with high levels of GRβ expression such as immune cells.

On the other hand the expression of the GRβ splice variants is very low in other tissues and cell-types[24]. Therefore, its role in other tissues may be limited. Possible involvement of the GRβ splice variant in the regulation of the HPA-axis is unexplored. Our ex vivo data are in accordance with this finding, but CRP levels in this population were not significantly associated with *GR-9*β. However the trend towards higher CRP levels in haplotype3 carriers compared to non-carriers warrants further investigation in a larger cohort.

While haplotype 5 is associated with reduced GC-sensitivity through parameters supposedly influenced by GR-mediated transactivation, haplotype3 appears to be associated with reduced GC-sensitivity via GR-mediated transrepression.

Future studies on transrepressive effects of the GR such as other inflammatory parameters and diseases as well as further studies on the mechanistic background are needed.

Understanding these differential effects on transactivation and transrepression is crucial for understanding differences in glucocorticoid sensitivity of patients, their susceptibility to diseases and their response to steroid treatment.

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Chapter 3

Staphylococcus aureus nasal carriage isassociated with glucocorticoid receptor gene polymorphisms

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ABSTRACT

The aim of this study was to determine whether polymorphisms of the glucocorticoid receptor gene, influencing glucocorticoid sensitivity, are associated with persistent nasal carriage of *S. aureus.* Of 2929 participants two nasal swab cultures were obtained. Subjects were classified as persistent carriers (n = 563) if both cultures were positive. GG-homozygotes of the exon 9beta polymorphism were associated with a 68% reduced risk of persistent *S. aureus* nasal carriage, while carriers of the codon 23 Lys-allele displayed an 80% increased risk. Thus, genotype dependent variation in the sensitivity to glucocorticoids is associated with tolerance towards staphylococcal nasal colonization.
Key words: S.aureus nasal carriage, colonization, glucocorticoid receptor, gene, polymorphism

INTRODUCTION

The anterior nares are the primary ecological reservoir of *Staphylococcus aureus* in humans and *S. aureus* nasal carriage is a major risk factor for a variety of infections[1]. Three human nasal *S. aureus* carriage patterns can be distinguished: persistent-, intermittent- and non-carriage. *S. aureus* density in the anterior nares is highest in persistent carriers[2], which may explain their increased risk of developing *S. aureus* infections. Variation among colonizing strains is higher for intermittent carriers, suggesting that the basic host determinants of persistent and intermittent carriage are different. The biology of nasal colonization with *S. aureus* remains incompletely understood. A variety of bacterial factors have been deemed important for maintaining colonization by *S. aureus* of the human nasal cavity. Besides polysaccharides, proteins belonging to the group of microbial surface components recognizing adhesive matrixmolecules play a role in bacterial adhesion[1]. In addition, environmental factors, as well as host factors of the immune status are thought to play a pivotal role in determining the *S. aureus* nasal carrier state[3].
Immune suppression by glucocorticoids is well known in the treatment of many inflammatory and autoimmune diseases or organ transplantation. The general cortisol status of individuals has been suggested as an important determinant of infection susceptibility. Glucocorticoids repress a large number of pro-inflammatory cytokines, activate a number of anti-inflammatory genes and up-regulate cell adhesion molecules such as ICAM-1[4], while down-regulation of neutrophil adhesion molecules has also been documented[5]. The ability of *S. aureus* to evade the immune response of the host by surviving inside neutrophils has been shown to be a virulence factor[6]. Impaired phagocytic activity is probably also a central factor in determining *S. aureus* nasal carriage[7]. Several glucocorticoid receptor (GR) gene polymorphisms are thought to be functional and have been described to be associated with variation in glucocorticoid sensitivity, variation in insulin sensitivity, changes in body fat distribution and with autoimmune diseases such as rheumatoid arthritis[8, 9]. Consequently, changes in glucocorticoid sensitivity may predispose to or protect from microbial colonization or infection on the one hand, or to autoimmune disease on the other.
We searched for host immuno-genetic markers for *S. aureus* nasal carriage. In the present study we aimed to determine whether the 4 known functional polymorphisms of the GR gene are associated with persistent *S. aureus* nasal carriage.
Materials & Methods

Study population

This study was conducted as part of the Rotterdam Study, a prospective, population-based cohort study on determinants of disease and disability in elderly persons started

in Rotterdam, The Netherlands in 1990 among 7983 participants[10]. The second follow-up of this study was performed between April 1st 1997 and December 31st 1999 among 4,797 remaining participants. No selection was applied to these subjects. The Medical Ethics Committee of the Erasmus MC, approved the study. Informed consent was obtained from all participants. Two quantitative *S. aureus*-specific nasal swab cultures with one-week intervals were performed[2]. We obtained complete bacteriological results in 3,851 persons, 2,224 females and 1,627 males. Subjects were classified as persistent carriers (n = 678) if both cultures were positive. Those with both cultures negative were classified as non carriers (n = 2804)[2].

Genotyping

All participants were genotyped for 4 known functional GR gene polymorphisms. We used the genotype data for each of the 4 polymorphisms to infer the haplotypes, based on Bayesian linkage disequilibrium analyses[11](Figure 1). Per haplotype three genotype combinations were distinguished, carrying 0, 1 or 2 copies of the haplotype allele (Figure 1). Haplotype 2 is characterized by the G allele of the Bcl-1 polymorhism, identified as an intronic C-to-G nucleotide substitution 646 nucleotides downstream from exon 2 (no rs number)[12]. Haplotype 3 is characterized by the G allele of the nucleotide substitution

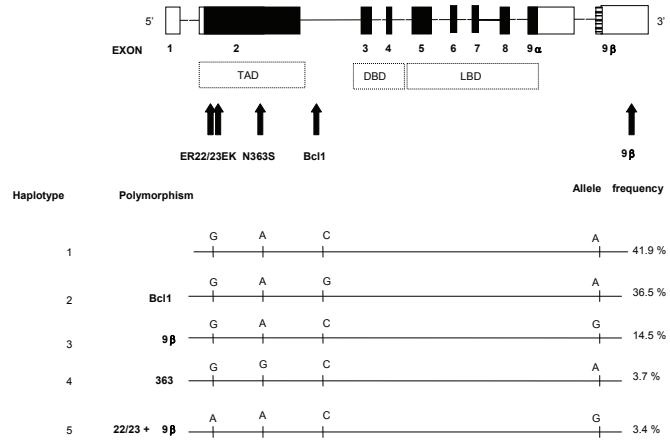

Figure 1 Schematic overview of the glucocorticoid receptor (GR) gene polymorphisms and genotypes. The alleles were defined as haplotypes such as "G-A-C-A" (haplotype 1) representing a Guanidine (G) nucleotide at the 200 G>A polymorphic site, an Adenosine (A) nucleotide at the 1220 A>G polymorphic site, a Cytidine (C) nucleotide at the C>G Bcl1 polymorphic site and an Adenosine (A) at the A>G 9Beta polymorphic site. TAD = transactivating domain, DBD= DNA binding domain, LBD= ligand binding domain.

located in the 3' end of exon 9β, which encodes for the 3' UTR of the mRNA of the hGRβ isoform (nucleotide 3669 in X03348; rs 6198 in http://www.ncbi.nlm.nih.gov/SNP))[13].
Haplotype 4 is characterized by the G-allele of the *N363S* polymorphism of exon 2 (nucleotide 1220 in NM_000176, rs 6195), located in the transactivation domain[14]. Haplotype 5 is characterized by the A-allele of the *ER22/23EK* polymorphism, which is a combination of two linked single-nucleotide variations in codons 22 and 23 (nucleotides 198 and 200; rs 6189 and rs 6190, respectively), leading to an arginine-to-lysine change in codon 23 in the transactivation domain[8], in combination with the G allele of the 9beta polymorphism that is also present in haplotype 3.
DNA was isolated from peripheral blood leukocytes using standard techniques, and stored at -20^{0}C. PCR amplification and genotyping were performed using 5 ng genomic DNA for the Taqman allelic discrimination assay. Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems. For details see http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism7900HT (Applied Biosystems, Foster City, CA, USA) in 384 wells format.

Statistical analysis

To compare baseline characteristics between the complete Rotterdam Study cohort, and participants with a complete dataset on *S. aureus* nasal carrier state, Pearson's Chi-square test was used for dichotomous variables and Mann-Whitney's test or Kruskal-Wallis' test were used for continuous variables. Associations between the GR genotypes, defined by 0,1 or 2 copies of the haplotype allele, and *S. aureus* nasal carrier status were first analyzed univariately using Pearson's Chi-square test. Multivariate logistic regression analysis was then employed to correct for the covariates age, gender, smoking (coded as never, past, and current), eczema (coded yes or no) and fasting serum glucose levels. Analysis for dominant, recessive or allele dose effects was performed by grouping subjects by allele copy number for all haplotype alleles, while correcting for covariates. Thereafter, the 15 possible genotypes were entered into the logistic regression model, again correcting for covariates. Results are reported as odds ratios with 95 percent confidence intervals. Two-sided *P* values of less than 0.05 were considered statistically significant.

RESULTS

For 2,929 participants a complete dataset on *S. aureus* nasal carrier state, confounding variables and all GR gene polymorphisms were available. Median age was 72 years, 58% were women, 18% were current and 48% were past smokers and mean fasting glucose was 5.9 mmol/L (Table 1). These baseline characteristics were not significantly different

Table 1. Baseline characteristics of the study population by genotype

genotype, no. of haplotype copies	gender	age	glucose level	eczema no. (%)	Smoking status, No. (%)		
	male no.(%)	Mean years (sd)	mmol/l (sd)	yes	non	past	current
Bcl1							
0	490 (42)	72,1 (6,9)	5,9 (1,5)	111 (9)	382 (32)	575 (49)	221 (19)
1	588 (43)	72,6 (6,8)	5,9 (1,4)	131 (10)	486 (36)	647 (47)	231 (17)
2	162 (42)	72,0 (6,8)	5,9 (1,4)	39 (10)	135 (35)	192 (50)	60 (15)
9β							
0	853 (43)	72,2 (6,7)	5,9 (1,5)	186 (10)	658 (33)	974 (50)	335 (17)
1	343 (39)	72,5 (7,0)	5,9 (1,4)	89 (10)	325 (37)	395 (45)	156 (18)
2	44 (51)	72,8 (8,0)	6,2 (1,9)	6 (7)	20 (23)	45 (52)	21 (25)
Ans363Ser							
0	1154 (43)	72,3 (6,9)	5,9 (1,5)	263 (10)	930 (34)	1313 (48)	474 (18)
1	85 (41)	72,2 (6,3)	5,8 (1,2)	18 (9)	72 (34)	100 (48)	37 (18)
2	1 (33)	71,5 (6,1)	5,2 (0,5)	0	1	1	1
ER22/23EK + 9β							
E 0	1165 (43)	72,3 (6,8)	5,9 (1,5)	265 (10)	935 (34)	1330 (49)	472 (17)
1	72 (39)	72,6 (7,1)	5,9 (1,4)	16 (9)	67 (36)	82 (44)	38 (20)
2	3(60)	70,6 (7,8)	6,6 (2,4)	0	1	2	2

from those for the complete Rotterdam Study cohort. Prevalence of persistent *S. aureus* nasal carriage in the final cohort was 19.2% (563/2929).

In non *S. aureus* carriers, the distribution of genotypes for all individual GR gene polymorphisms were in Hardy-Weinberg equilibrium (p>0.05; Table 2). The frequencies of haplotype alleles 1-5 were 41.9%, 36.5%, 14.5%, 3.7% and 3.4% respectively (Figure 1).

No associations were found between GR haplotypes and the covariates age, gender, smoking, eczema and fasting serum glucose levels.

Genotypes defined by carrier status for haplotype 2 and 4 showed no significant difference in the prevalence of *S. aureus* nasal carriage. Analysis of the association of the five GR haplotypes revealed that haplotype 3 was significantly associated with a lower prevalence of *S. aureus* nasal carriage with evidence for a recessive effect. After correction for age, gender, fasting glucose, eczema and smoking habit by logistic regression analyses, this association remained essentially unchanged. Haplotype 3 homozygotes had a 68% lower risk of persistent *S. aureus* nasal carriage compared to heterozygous or non-carriers of haplotype 3 (odds ratio 0.32; 95% confidence interval (0.13-0.82)). Haplotype 5 carriers (heterozygotes+homozygotes) were associated with a higher prevalence of *S. aureus* nasal carriage, having a 38% higher risk of persistent *S. aureus* nasal carriage compared to non-carriers (odds ratio 1.38; 95% confidence interval (0.97-1.97).

Table 2. The distribution of glucocorticoid receptor gene genotypes and S. aureus nasal carriage status.

Genotype by no of haplotype copies	HWE [a] P-value	N-SNC [b] n (%)	P-SNC [c] n (%)	Total n	Univariate analysis [d] OR (95% CI)	Multivariate analysis [e] OR (95% CI)	Change in risk of colonization
Haplotype 1:	0,2	2,366 (80.8)	563 (19.2)	2,929			
2		423 (79.7%)	108 (20.3%)	531	1 (reference)	1 (reference)	
1		1,117 (80.1%)	278 (19.9%)	1,395	0.86 (0.70-1.06)	0.85 (0.68-1.04)	
0		826 (82.4%)	177 (17.6%)	1,003	0.84 (0.64-1.10)	0.85 (0.65-1.11)	
Haplotype 2:	0,5	2,366 (80.8)	563 (19.2)	2,929			
0		945 (80.2)	233 (19.8)	1,178	1 (reference)	1 (reference)	
1		1,099(80.6)	265 (19.4)	1,364	0.98 (0.80-1.19)	0.97 (0.80-1.18)	
2		322 (83.2)	65 (16.8)	387	0.82 (0.61-1.11)	0.80 (0.59-1.08)	Not significant
Haplotype 3:	0,94	2,366 (80.8)	563 (19.2)	2,929			
0		1,725 (80.5)	418 (19.5)	2,143	1 (reference)	1 (reference)	
1		581 (80.6)	140 (19.4)	721	1.00 (0.80-1.23)	1.01 (0.81-1.25)	
2		60 (92.3)	5 (7.7)	65	0.34 (0.14-0.86)	0.32 (0.13-0.82)	68% reduction
Haplotype 4:	0,81	2,366 (80.8)	563 (19.2)	2,929			
0		2,197 (80.9)	520 (19.1)	2,717	1 (reference)	1 (reference)	
1		166 (79.4)	43 (20.6)	209	1.09 (0.77-1.55)	1.13 (0.80-1.61)	Not significant
2		3 (100)	0 (0)	3	na	na	
Haplotype 5:	0,16	2,366 (80.8)	563 (19.2)	2,929			
0		2,218 (81.0)	519 (19.0)	2,737	1 (reference)	1 (reference)	
1		143 (76.5)	44 (23.5)	187	1.32 (0.93-1.87)	1.38 (0.97-1.97)	Not significant
2		5 (100)	0 (0)	5	na	na	
Haplotype combination 1 & 5	0,26	2,366 (80.8)	563 (19.2)	2,929			
No		2,310 (81.0%)	541 (19.0%)	2,851	1 (reference)	1 (reference)	
Yes		56 (71.8%)	22 (28.2%)	78	1.68 (1.02-2.77)	1.80 (1.08-3.00)	80% increase

[a] Hardy Weinberg equilibrium, [b] non S. aureus nasal carrier, [c] persistent S. aureus nasal carrier, [d] Univariate: Pearson Chi-Square test, [e] Adjusted for age, gender, smoking, eczema and fasting serum glucose.

This association was even stronger when investigating all potential genotypes, i.e., all haplotype allele combinations (i.e., 15 genotypes), by logistic regression analysis. The genotype combination of the haplotype 5 and the haplotype 1 allele was significantly

associated with *S. aureus* nasal carriage. This latter genotype was shown to increase the risk of persistent *S. aureus* nasal carriage by 80% (odds ratio 1.80; 95% confidence interval, 1.08-3.00). No direct relation was found between fasting blood glucose levels and any of the haplotypes investigated.

DISCUSSION

Our study for the first time suggests that also human DNA polymorphisms as found in the GR gene are significantly associated with *S. aureus* nasal carriage status.
The four GR gene polymorphisms that we studied, have all been associated with variation in glucocorticoid sensitivity[8]. Haplotype 2, previously found to be associated with increased glucocorticoid sensitivity[12], was not associated with *S.aureus* carrier status.
We found that homozygous presence of haplotype 3 conferred a 68% lower risk of persistent *S.aureus* nasal carriage. This G-allele of the exon 9beta polymorphism is over-represented in a group of patients with rheumatoid arthritis[9]. The mechanism for this is unknown, but stabilization of the GRbeta mRNA by the presence of this G-allele – as indeed observed in vitro [9, 13] – may play a role. The G-allele has an interrupted mRNA destabilizing ATTTA motif, resulting in a higher stability for the GRbeta mRNA. This stabilization induces relative glucocorticoid insensitivity through accumulation of the GRbeta protein which has been reported to have a dominant negative influence on GRalpha action[9, 13]. This in turn may lead to an immune enhancement-, predisposing to chronic autoimmune inflammatory disease such as rheumatoid arthritis, while protecting from *S.aureus* colonization. Although no data on insulin sensitivity or tissue glucose concentrations are available for the population studied, the lack of association between fasting blood glucose and the exon 9beta polymorphism suggests that a reduced glucocorticoid-induced immune suppression is the most likely explanation for the association observed here.
Haplotype 4 has been shown to increase glucocorticoid sensitivity[14] . It was, however, not associated with *S. aureus* carriage.
Haplotype 5 has been associated with increased resistance to glucocorticoids, higher cortisol levels after dexamethasone suppression testing, lower fasting insulin concentrations and better insulin sensitivity[8]. We found that carriers of this haplotype allele were at an increased risk of persistent *S. aureus* carriage. Persons with the genotypic combination of haplotype 1 together with this haplotype allele had an 80% higher risk of persistent *S. aureus* carriage versus all other genotypes (OR 1.8; 95%CI 1.08-3.00). Haplotype 5 carriers show a relative glucocorticoid resistance due to less transactivation in combination with normal transinhibition of the pro-inflammatory nuclear factor NF-κB[15]. We hypothesize that the higher cortisol levels in these carriers lead to an increased transinhibition and thus to suppression of the immune system. It should be noted, that

in haplotype 5 (with increased risk for SA carriage) the ER2223EK polymorphism is combined with the G-variant of 9beta. While a protective effect of this G-allele 9beta variant was seen in homozygotes for this haplotype 3. This suggests a recessive effect of the 9beta G-allele. Second, all five 23Lys homozygotes were non-carriers of *S. aureus*, which might be due to the fact that they are also 9beta G-allele homozygous.

The current availability of high-tech, high-throughput genotyping technologies in combination with the availability of a large prospective population-based epidemiologic survey has provided a unique opportunity to study genetic determinants of *S. aureus* nasal carriage. The limitation is that our study has a descriptive nature and future research should lead to better insight in the underlying mechanisms. In addition, our study population consisted of an elderly population (>55 years old) and of more than 98% Caucasians. Therefore, results might not apply to groups of different genetic or environmental backgrounds. Further, little is known on the physiology of the GR gene polymorphisms. It was recently demonstrated that glucocorticoids, in conjunction with pro-inflammatory cytokines, affect the expression of toll-like receptors, the main pattern recognition molecules for gram positive pathogens such as *S. aureus*[16]. Whether the various GR gene polymorphisms studied here are (partially) defective in supporting this biological activity is currently unknown, but, again, warrants further investigation.

Our data suggest that genetic variation in the GR gene affects persistent *S. aureus* nasal carriage. Further investigations would form a *de novo* field of research focusing on *S. aureus* carriage and glucocorticoid/ GR gene dependent innate immune determinants. Apparently genotype dependent variation in the sensitivity to glucocorticoids seems to influence the individual status of immune suppression. These immunomodulatory properties of glucocorticoid hormones can be important in the prevention and treatment of many diseases.

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Chapter 4

Glucocorticoid receptor gene and risk of cardiovascular disease.

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ABSTRACT

Background: Genetic variants in immuno-modulating genes have been suggested to contribute to risk of cardiovascular disease. Glucocorticoids are important regulators of inflammatory processes and the immune system. Our aim was to determine the contribution of genetic glucocorticoid receptor variants, with different cortisol sensitivities, to the risk of cardiovascular disease.

Methods: The study was conducted in a large (n=7983) population-based, prospective cohort of the Rotterdam Study. The mean follow up duration was 8.9 years. Measures of cardiovascular disease were incident myocardial infarction, coronary heart disease, high sensitive C-reactive protein, interleukin-6 and arteria carotis intima media thickness.

Results: Persons, homozygous for haplotype 3, which is a common variant of the glucocorticoid receptor gene, had a more than twice increased risk of myocardial infarction (hazard ratio 2.2, 95%CI 1.15-4.15), and an almost three times increased risk of coronary heart disease (hazard ratio 2.8, 95%CI 1.52-5.24) as compared to non-homozygous persons. In addition, their levels of C-reactive protein, interleukin-6 and carotis intima media thickness were higher. No associations were found for the other haplotypes.

Conclusions: The glucocorticoid receptor gene haplotype 3 is a common genetic variant and is related to a more active pro-inflammatory system. This haplotype is associated with risk of cardiovascular disease and its parameters. These results should be taken as hypothesis generating until they have been replicated in other studies. Our findings suggest that genetically determined cortisol sensitivity is involved in the pathogenesis of cardiovascular disease and might identify a subgroup at risk.

INTRODUCTION

Genetic susceptibility for cardiovascular disease has been found for diverse systems, such as the hemostatic system, the metabolism of lipids and glucose and in the action of sex hormones[1, 2]. Recently, interest in immuno-genetic susceptibility for atherosclerosis has been increasing because inflammation and immune response have been found to contribute to atherogenesis[3]. Several proteins of the innate immune response are found to predict cardiovascular risk, the most important being C-reactive protein[3, 4].

Glucocorticoids are important regulators of the immune system, inflammatory processes and many other processes involved in fat and glucose metabolism and the cardiovascular system. Several studies showed that high levels of glucocorticoids result in unfavourable cardiovascular risk factors, e.g. visceral obesity, steroid-induced diabetes and hypercholesterolemia[5]. Glucocorticoids exert their effect through the glucocorticoid receptor which is expressed in most cells of the human body and regulates the expression of multiple genes[5]. The sensitivity to glucocorticoids varies considerably between individuals[6]. Polymorphisms in the glucocorticoid receptor gene are thought to play a role in this[7]. Four glucocorticoid receptor gene variants have been associated with a change in cortisol sensitivity: BclI, *N363S*, *ER22/23EK* and *GR-9*β[7]. Three of these polymorphisms (*ER22/23EK*, *N363S* and BclI) have been studied in a population of 552 elderly for their risk on cardiovascular disease and no association was found[8]. However, literature reports are conflicting[9]. Replication or validation of findings from genetic association studies is needed.

In recent papers we reported on the earlier mentioned four glucocorticoid receptor gene variants and their haplotypes[10, 11] (Figure1). Our finding of decreased immuno-suppression in haplotype3 (*GR-9*β polymorphism) carriers[10, 11] led to the a-priori hypothesis that these persons might have elevated inflammatory parameters and an increased risk of cardiovascular disease, through a lifelong exposure to diminished cortisol suppression of the pro-inflammatory system. Our aim was to examine the association of glucocorticoid receptor variants, which have been associated with changes in cortisol sensitivity, with different measures of atherosclerosis, like hs-C-reactive protein levels, interleukin-6 levels, carotis intima media thickness, risk of myocardial infarction and coronary heart disease. This was examined in a large population based follow-up study.

METHODS

Study population

This study was conducted as part of the Rotterdam Study, a population-based prospective cohort study on determinants of disease and disability in 7983 persons, aged 55 years and older, living in Rotterdam, The Netherlands[12]. Our study population included men and women aged 55 years and older, who were of Caucasian origin, were able to visit the research center and who completed all parts of the baseline examination including providing a blood sample. The Medical Ethics Committee of the Erasmus Medical Center, approved the study. Informed consent and permission to retrieve information from treating physicians was obtained from all participants.

Measurements

A trained interviewer visited all subjects at home and collected information on current health status, medical history, drug use, and smoking using a computerized questionnaire. Cardiovascular risk factors were obtained by interview and physical examination at baseline as described before[1]. A history of myocardial infarction was considered present in case of a self-report of myocardial infarction, confirmed by ECG or additional clinical information, or the presence of an ECG characteristic of prior myocardial infarction. Interview information included smoking habits, age at menopause and medication including use of hormone replacement therapy (HRT). Smoking was categorized as current, past or never smoker. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Hypertension was defined as a systolic pressure ≥ 160 mmHg and/or a diastolic pressure ≥ 100 mmHg and/or use of antihypertensive medication, encompassing grade 2 and grade 3 hypertension according to the World Health Organization (WHO) criteria[13]. After an overnight fast, blood samples were obtained. Diabetes mellitus was considered present with current use of antidiabetic medication or a non-fasting or postload glucose level of 198 mg/dL (11 mmol/L) or higher according to the WHO criteria[14]. As a measure of atherosclerosis, intima media thickness (millimetre) was measured by ultrasound as described before[15].

Serum, stored at –20 °C, was used for measurement of high sensitivity CRP (hs-CRP) by a nephelometric method (Dade-Behring) as described elsewhere[16]. The detection limit of the assay was 0.2 mg/dL; the inter- and intra-assay coefficient of variation for the method used were both 3.2%. Serum levels of IL-6 were assessed in a random sample of 714 participants by using commercially available ELISA (IL-6, Quantikine HS).

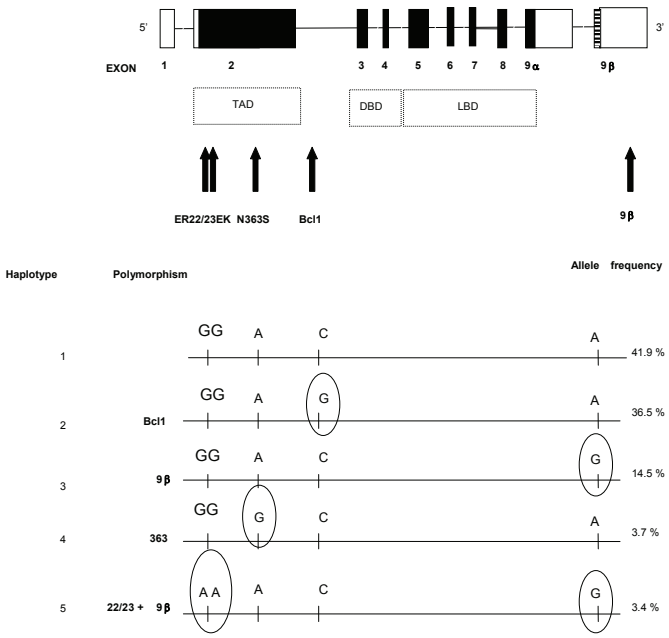

Figure 1 Schematic overview of the glucocorticoid receptor (GR) gene polymorphisms and haplotypes. Haplotypes are numbered in order of decreasing frequency. The nucleic acid changes are indicated; C= cythidine, G= Guanidine, A= Adenosine. TAD = transactivating domain, DBD= DNA binding domain, LBD= ligand binding domain.

Genotyping

The SNP database dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) currently lists 461 SNPs in the human GR gene locus. However, of these polymorphisms only 15 are in the coding region. And of these only 8 confer changes in amino acid. We chose to genotype the 4 polymorphisms in the glucocorticoid receptor gene that have been associated with changes in glucocorticoid sensitivity only.

DNA was isolated from peripheral blood leukocytes using standard techniques, dissolved in double-distilled water and stored at -20^{0}C. PCR amplification and genotyping were performed using 5 ng genomic DNA for the Taqman allelic discrimination assay. Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems. For details see http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism7900HT (Applied Biosystems, Foster City, CA, USA) in 384 wells format.

All participants were genotyped for 4 known glucocorticoid receptor gene polymorphisms. Figure 1 schematically depicts this gene and the location of the 4 polymorphisms, their specific nucleotide variations and allele frequencies. The location of gluco-

corticoid receptor gene polymorphisms *ER22/23EK* (rs 6189 and 6190), *N363S* (rs 6195) and Bcl-1 (no rs number) have been previously described[7]. The *GR-9*β polymorphism (nucleotide 3669 in X03348; rs 6198) is an A to G nucleotide substitution, located in the 'ATTTA' motif in the 3'-UTR of the gene.

We used the genotype data for each of the 4 polymorphisms to infer the haplotypes present in the population using the program PHASE which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data[17]. Three of the four polymorphisms were found to be mutually exclusive, only the codon 23 A-allele was always present in combination with the *GR-9*β G-allele. The alleles were defined as haplotypes such as "GG-A-C-A" (haplotype1) representing a Guanidine (G) nucleotide at the 198 and 200 G>A polymorphic site (rs 6189 and 6190 respectively), an Adenosine (A) nucleotide at the 1220 A>G polymorphic site (rs 6195), a Cytidine (C) nucleotide at the C>G *Bcl1* polymorphic site and an Adenosine (A) at the A>G *GR-9*β polymorphic site (rs 6198)(Figure 1). Per haplotype three genotype combinations were distinguished, carrying 0, 1 or 2 copies of the haplotype allele. Haplotype1 carries the major alleles of the polymorphisms; therefore, the reference allele is defined as carrying 2 copies of haplotype 1.

Follow up procedures

Follow up data on myocardial infarction (MI) and coronary heart disease (CHD) were collected from baseline (1990-1993) until January 1, 2003. General practitioners in the Rotterdam area by means of a computerized system reported cardiovascular disease morbidity and mortality. In the case of recurrent MI or CHD during follow up, the first event was used in the analysis. All reported research physicians who collected information from the patients' medical records verified events. Consulting hospital discharge letters from the medical specialist also identified this information and information on revascularization procedures. All events were coded independently by two research physicians according to the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10)[18]. Finally, a medical expert in cardiovascular disease reviewed all coded events for final classification. All available information, including ECG, cardiac enzymes and the clinical judgement of the treating specialist, were used to code the events. In the present study, we used the following endpoints: incident MI (I21) and CHD. CHD was defined as MI (I21), percutaneous transluminal coronary angioplasty (PTCA; Z95.5), coronary artery bypass graft (CABG; Z95.1) and death from CHD (I20-I25).

Population for analysis

A total of 7129 participants, visited the research center. DNA was available for 6571 subjects. Reasons for non-availability of DNA were failure of venapuncture, failure of DNA isolation in the laboratory or failure of allelic discrimination by taqman analysis (approximately 5% per polymorphism), or incomplete haplotypes (7%). For only 7 persons follow-up data on coronary heart disease were missing.
Genotyping of all 4 polymorphisms was successful in 6081 subjects. For 6074 of these subjects follow up data on CHD were complete. Persons with a history of MI at baseline were excluded (n=1196). In total, 4878 subjects were available for analysis of the association between haplotype and incident CHD. For hs-CRP and IL-6 analysis, subjects with possible acute inflammation as indicated by hs-CRP > 10 mg/dL or IL-6 levels >10 pg/mL were excluded. In the study population, 5.0% of the hs-CRP and 2.5% of the IL-6 levels were >10 mg/dL.

Statistical analysis

Differences in baseline characteristics between between carriers of 0,1 and 2 copies of haplotype1 to 5 or between men and women were examined by one-way analysis of variance (ANOVA) (continuous variables) and Pearson's chi-square (dichotomous variables). Because of the low numbers of homozygous subjects for haplotype4 (n=6) and 5 (n=8), these haplotypes were analysed as carriers (1 or 2 copies) and non carriers (0 copies). Results are reported as means with standard errors. We used logarithmically transformed values of CRP and IL-6 to normalize the distribution of these variables. The association between the glucocorticoid receptor haplotypes and CHD events was evaluated by age adjusted Cox proportional hazards model. Hazard ratios were computed as estimates of relative risk. To account for possible confounding, we excluded all subjects with previous MI at baseline (model 1) and computed relative risks in a multivariate model containing the following variables: age and sex (model 1), with additionally body mass index (BMI), systolic blood pressure, smoking, diabetes mellitus, total and HDL-cholesterol (model 2), and hs-CRP (model 3). The cardiovascular risk factors (model 2) were also used in the analysis of cumulative risk by adding them as covariates in the cox regression test (Fig2). In the sexe specific analyses age at menopause and use of HRT were added to model 2 for women. Differences in hs-CRP and IL-6 levels between haplotypes were examined by ANOVA, with Bonferroni correction for multiple testing, and corrected for age, bmi and smoking. Carotis intima media thickness, with a skewed distribution, was tested by a non-parametric test (Kruskal-Wallis). Missing values did not exceed 3.5% for any covariate. For all statistical analyses, p-values below 0.05 were considered to indicate

statistically significant differences. Data were analyzed using SPSS 12.0.1 for windows (SPSS Inc., Chicago, USA).

RESULTS

Baseline characteristics of the study population are described in Table 1. The median age was 68.5 years, 3006 (62%) were women. Frequencies of the haplotype alleles are presented in Figure 1. Due to their high linkage disequilibrium, 3 of the 4 described polymorphisms are mutually exclusive. The distribution of genotypes for all glucocorticoid receptor gene polymorphisms was in Hardy-Weinberg equilibrium (p>0.05). Hap-

Table 1. Baseline characteristics of the study population

	Women	Men
Numbers	3006 (62%)	1872 (38%)
Age (years)	69.2 ± 9.2	67.4 ± 7.9
BMI (kg/m2)	26.6 ± 4.0	25.6 ± 2.8
Diastolic blood pressure (mmHg)	73 ± 11	75 ± 11
Systolic blood pressure (mmHg)	139 ± 23	139 ± 22
Smokers (%)		
Never	54.3%	8.6%
Current	18.2%	30.3%
Past	27.5%	61.1%
Hypertension (%)	35.5%	27.2%
Diabetes Mellitus (%)	10.0%	7.9%
Total cholesterol (mmol/L)	6.8 ± 1.2	6.3 ± 1.2
HDL-cholesterol (mmol/L)	1.5 ± 0.4	1.2 ± 0.3
Previous MI (%)	8.8%	17.3%
Age at menopause (years)	48.9 ± 4.9	
Ever use of HRT (%)	16.1%	

555 men and 641 women were excluded because of a history of MI at baseline. Values are expressed as mean plus/minus standard deviation or as percentages

lotype1, 2 and 3 were most frequent with allele frequencies of 41.9 %, 36.5 % and 14.5 %, respectively. Haplotypes 4 and 5 had allele frequencies of 3.7% and 3.4% respectively. Comparison of means of baseline characteristics between carriers of 0,1 and 2 copies of haplotype1to 5 revealed no significant differences for the covariates.

The mean follow-up duration was 8.9 years (standard deviation 3.2 years; range 0.05 to 13.5 years). During follow up, an incident MI occurred in 220 (4.5%) participants and 493 (10.1%) of the participants had a CHD event. MI and CHD events were more frequent in men (135 (7.2%) MI and 257 (13.7%) CHD events) compared to women (85 (2.8%) MI and

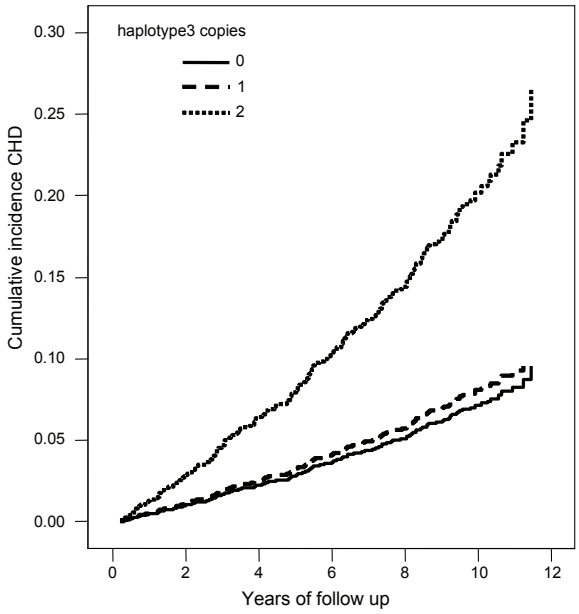

Figure 2 Cumulative risk of coronary heart disease for categories of glucocorticoid receptor haplotype 3 carriers.

Table 2a. Risk of myocardial infarction by glucocorticoid haplotype.

				Model 1[c]	Model 2[d]
	copies	n	events	HR (95%CI)	HR (95%CI)
haplotype 1	0	1828	82 (4.5%)	1 (reference)	1 (reference)
	1	2177	89 (4.1%)	0.92 (0.68-1.24)	0.89 (0.66-1.21)
	2	873	49 (5.6%)	1.28 (0.90-1.82)	1.22 (0.85-1.74)
haplotype 2	0	1934	93 (4.8%)	1 (reference)	1 (reference)
	1	2290	99 (4.3%)	0.90 (0.68-1.20)	0.92 (0.69-1.22)
	2	654	28 (4.3%)	0.87 (0.57-1.33)	0.92 (0.60-1.41)
haplotype 3	0	3575	161 (4.5%)	1 (reference)	1 (reference)
	1	1192	49 (4.1%)	0.91 (0.66-1.26)	0.90 (0.65-1.24)
	2	111	10 (9%)	1.93 (1.02-3.66)[a]	2.14 (1.13-4.07) [b]
haplotype 4	0	4511	209 (4.6%)	1 (reference)	1 (reference)
	1or2	376	11 (3.0%)	0.64 (0.35-1.17)	0.66 (0.36-1.21)
haplotype 5	0	4553	206 (4.5%)	1 (reference)	1 (reference)
	1or2	325	14 (4.3%)	0.96 (0.56-1.65)	0.94 (0.55-1.62)

HR=hazard ratio, 95%CI=95% confidence interval. [a] p=0.04, [b] p=0.02

[c] Multivariate model with covariables: age and sexe (model 1), and dadditionally with body mass index (BMI), systolic blood pressure, smoking, diabetes mellitus, total and HDL-cholesterol (model 2).

Table 2b. Risk of coronary heart disease by glucocorticoid receptor haplotype

	copies	n	events	Model 1 HR (95%CI)	Model 2 HR (95%CI)
haplotype 1	0	1828	194 (10.6%)	1 (reference)	1 (reference)
	1	2177	195 (9.0%)	0.85 (0.70-0.1.03)	0.77 (0.57-1.05)
	2	873	104 (11.9%)	1.16 (0.92-1.48)	1.22 (0.86-1.73)
haplotype 2	0	1934	204 (10.5%)	1 (reference)	1 (reference)
	1	2290	223 (9.7%)	0.93 (0.77-1.12)	0.88 (0.66-1.17)
	2	654	66 (10.1%)	0.95 (0.73-1.27)	0.82 (0.53-1.28)
haplotype 3	0	3575	355 (9.9%)	1 (reference)	1 (reference)
	1	1192	119 (10.0%)	0.98 (0.80-1.21)	1.14 (0.84-1.55)
	2	111	19 (17.1%)	1.65 (1.04-2.62) [a]	2.60 (1.40-4.81) [b]
haplotype 4	0	4511	466 (10.3%)	1 (reference)	1 (reference)
	1or2	367	27 (7.4%)	0.70 (0.48-1.04)	0.75 (0.42-1.34)
haplotype 5	0	4553	459 (10.1%)	1 (reference)	1 (reference)
	1or2	325	34 (10.5%)	1.03 (0.73-1.46)	0.78 (0.44-1.41)

HR=hazard ratio, 95%CI=95% confidence interval. [a]p= 0.03; [b]p= 0.002

236 (7.8%) CHD events. No association with MI or CHD was observed for glucocorticoid receptor haplotypes 1, 2, 4 or 5 (Table 2). Persons, homozygous for haplotype3 had an increased risk of MI (p=0.04) as well as CHD (p=0.03) compared to non-homozygous (Table 2). Adjustment for age and sex (model 1), and subsequently for BMI, systolic blood pressure, smoking, diabetes mellitus, total and HDL-cholesterol (model 2) strengthened these associations (p-value 0.02 for MI and 0.002 for CHD, Table 2). Figure 2 presents the cumulative risk of incident coronary heart disease during follow-up for persons carrying 0,1 or 2 copies of haplotype3, adjusted for cardiovascular risk factors.

Additional correction for hs-CRP in model 2 slightly lowered the risk of MI or CHD (hazard ratio 2.10 for MI and 2.73 for CHD). Risk of CHD remained significantly elevated after exclusion of incident MI (hazard ratio 3.3 (95%CI 1.42-7.68, p=0.005)). In sex-specific subgroup analyses the numbers of events were too small to justify statistical interpretation.

Hs-CRP, interleukin-6 and intima media thickness were significantly higher in homozygous carriers of haplotype3 (Table 3). No association was found between haplotype 3 and frequency of diabetes mellitus (data not shown). No association was observed for hs-CRP, interleukin-6 or intima media thickness and haplotypes 1,2, 4 or 5 (data not shown).

Table 3. C-reactive protein levels, interleukin-6 levels and carotis intima media thickness for glucocorticoid receptor haplotype3.

		N	Mean	SEM	p-value
hs-CRP	0	4020	2.4	0.03	
	1	1330	2.4	0.06	
	2	121	2.8	0.20	0.03 [a]
IL-6	0	434	2.2	0.07	
	1	166	2.6	0.14	
	2	10	3.1	0.72	0.01 [a]
IMT	0	2979	1.03	0.00	
	1	975	1.03	0.01	
	2	91	1.09	0.02	0.01 [b]

hs-CRP= high sensitive C-reactive protein (mg/dL), IL-6= Interleukin-6 (pg/mL), IMT=intima media thickness of arteria carotis (mm). [a]p-values from ANOVA, ln transformed, Bonferroni correction for multiple testing, corrected for age, body mass index and smoking. [b]p-value by non-parametric test (Kruskal Wallis)

DISCUSSION

In this large population-based follow-up study we found a common glucocorticoid receptor haplotype to be associated with increased risks of myocardial infarction and coronary heart disease. Persons, homozygous for haplotype3, had a 2.2 times increased risk of MI and a 2.8 times increased risk of CHD compared to non-homozygous. The increase in risk prediction achieved with haplotype3 was similar to that reported for other risk markers like cholesterol or hs-CRP[19]. In addition, homozygous carriers of haplotype3 showed significantly higher levels of hs-CRP, interleukin-6 and carotis intima media thickness (Table 3). No associations were found for the other haplotypes.

One of the major limitations of genetic association studies is lack of reproducibility. Although the numbers of events for MI and CHD in our study are limited, the results are further supported by the significant association that was found for hs-CRP and interleukin-6 levels as important inflammatory parameters and by intima media thickness as a measure of atherosclerosis (Table 3). These results were in line with our a-priori hypothesis based on previous studies. The *GR-9*β polymorphism of haplotype3 is located in the 3'-UTR end of the glucocorticoid receptor gene, which is transcribed in the GRβ splice variant thought to have a dominant negative effect on GRα[20]. In vitro data show that this polymorphism leads to increased GRβ expression by a more stable GRβ mRNA transcript and thereby to relative glucocorticoid resistance. Indeed, persons carrying haplotype3 seem to have a decreased glucocorticoid transrepression with a normal transactivation[11]. The influence of glucocorticoids on the immune system is regulated through transrepression. Clinical data show that persons homozygous for haplotype3 had a reduced risk of bacterial colonization with *Staphylococcus aureus* in the nose[10] and might be

more susceptible to rheumatoid arthritis[21], which suggests that the *GR-9*β polymorphism can lead to a more active immune system. MI, CHD and rheumatoid arthritis all have an inflammatory pathogenesis. We hypothesize that glucocorticoid receptor haplotype3 leads to a higher risk of MI and CHD, through a lifelong exposure to diminished cortisol suppression of the pro-inflammatory system.

There are multiple pathways through which altered glucocorticoid receptor sensitivity could influence these inflammatory processes. Glucocorticoids inhibit the immune response and the pro-inflammatory response, for example by suppression of the synthesis of cytokines and inflammatory mediators such as NF-κB, which is a key regulator in the process of atherosclerosis. Thus, they act on a variety of immune cells to control inflammation. Moreover, glucocorticoids influence vasodilation, through decreased nitric oxide release, vascular permeability and migration of leukocytes across the endothelium[5], which are all important factors in inflammation and atherogenesis[3]. Secondly, the glucocorticoid receptor can regulate the expression of toll-like receptors[22], which are known to activate signaling pathways to inflammation and are associated with CRP and CHD[23].

Our finding of a relationship of a common glucocorticoid receptor genetic variant with risk of MI, CHD and other parameters of atherosclerosis might have important implications for our understanding of the inflammatory pathogenesis of CHD. Possibly, glucocorticoid receptor genotype analysis can identify a subgroup, which may in particular benefit from anti-inflammatory treatment.

Glucocorticoid receptor haplotypes 1,2,4 and 5 were not associated with risk of MI, CHD, nor with hs-CRP, interleukin-6 levels or intima media thickness. These findings replicate and further support the results of a previous study, performed in a population of 552 elderly persons[8].

The limitations of our study include the possibility of remaining selection bias. The population for analysis was reduced because only subjects with complete haplotypes were included. We do not expect a bias as a result of this. Subjects with previous MI were excluded to study incident MI. Inclusion of these subjects in analysis did not change the results (data not shown). Further confounding or multiple testing could influence results. Nevertheless, the associations persisted despite adjustment for known risk factors and correction for multiple testing. Our study population included >98% Caucasians and results might not apply to groups with different genetic or environmental backgrounds. One of the major limitations of genetic association studies in general has been the lack of reproducibility. Therefore, although the consistency of the findings in this study is remarkable, the results should be taken as hypothesis generating until they have been replicated in other studies. Finally, additional mechanistic studies are warranted.

In conclusion, the glucocorticoid receptor gene haplotype3, which is related to a diminished suppressive effect of cortisol on the pro-inflammatory system, is associated to risk of myocardial infarction, coronary heart disease. This is further supported by higher lev-

els of hs-CRP and Interleukin-6, which are important markers for cardiovascular disease and higher carotis intima media thickness as a measure of atherosclerosis. These findings may provide new insight in our understanding of the inflammatory pathogenesis of cardiovascular disease and might identify a subgroup at risk.

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Chapter 5

Strategies for the characterization of disorders in cortisol sensitivity

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ABSTRACT

Context:
The clinical presentation of abnormalities in glucocorticoid (GC) sensitivity is diverse, and therefore it is difficult to diagnose this condition.

Objective and Design:
The objective of the study was to develop strategies for the characterization of GC sensitivity disorders.

Setting:
The study was conducted in an outpatient clinic.
Patients: Nine patients with GC sensitivity disorders participated.

Interventions:
Sequence analysis of the GC receptor (GR), determination of GR number per cell, GR ligand-binding affinity, and GR splice regulation were performed in freshly prepared peripheral blood mononuclear lymphocytes and Epstein-Barr virus-transformed lymphoblasts. Cellular GC sensitivity was determined *ex vivo* by measuring the effect of dexamethasone on GC-induced leucine-zipper and IL-2 mRNA levels and on cell proliferation.

Results:
Differences in GR number per cell, GR affinity, GR splice variants, and effects on transactivation or transrepression of GC-sensitive genes were observed between patients and controls. Epstein-Barr virus transformation of lymphoblasts had no influence on GR affinity but increased the GR number 5-fold in healthy controls. In patients diagnosed as cortisol resistant, however, GR number after transformation was increased significantly less than 5-fold, whereas a higher GR number was observed in a patient suspected of cortisol hypersensitivity.

Conclusion:
This study illustrates several strategies to define abnormalities in GC sensitivity by describing nine patients with affected GC sensitivity, all with a unique clinical course and background.

INTRODUCTION

Glucocorticoids (GCs) are key hormones in metabolic and immunological homeostasis and regulate many physiological processes[1]. Cortisol concentration is tightly regulated by the hypothalamic-pituitary-adrenal (HPA)-axis feedback system and depends on neural and other stimuli[2, 3].

The extremes of variability in cortisol sensitivity can be divided in cortisol resistance (CR) and hypersensitivity (CH). So far, only one case of CH has been reported, diagnosed in a patient with Cushingoid manifestations, despite persistent hypocortisolemia[4]. CR was first described[5] as an inherited disorder characterized by hypercortisolism without Cushingoid features. The negative feedback on the HPA axis is reduced, due to diminished GC sensitivity, resulting in higher cortisol secretion by the adrenal glands to keep balance between need and production. However, adrenal production of androgens and mineralocorticoids (MCs) is also increased, causing the symptoms of CR: hypertension, hypokalemia, disturbed spermatogenesis, and infertility in men and acne, hirsutism, male pattern of baldness, oligomenorrhea, and infertility in women[5, 6]. In children, premature adrenarche was reported[7].

Decreased GC sensitivity is often caused by abnormalities in the GC receptor (GR) including decreased affinity for GCs[6-8], decreased receptor number[6, 8, 9], decreased receptor DNA binding[6, 10], receptor thermolability[11], impaired receptor translocation to the nucleus[12], or altered protein-protein interaction with coactivators[13]. An increased concentration of the GR-ß splice variant, a dominant-negative inhibitor of active GR-α, has also been reported to cause CR, but always as acquired rather than inherited[14, 15]. A GR-P splice variant is thought to increase GR-α activity[14].

The molecular basis of CR has been elucidated in six kindreds and three sporadic cases as caused by mutations in the DNA- or hormone-binding domain of the GR gene. However, several years ago, we reported five patients diagnosed with clinical and/or biochemical CR, each with very diverse clinical presentations, without GR gene alterations[16].

For the present study, we invited nine patients with abnormal GC sensitivity. One patient hyperreacted to GC medication, whereas the others were diagnosed as CR. Of the latter group, three patients had been previously reported with mutations in the GR gene[17-19] and two patients without genetic GR alterations[16]; the other three patients were recently diagnosed and have not been described previously. The aim of our study was to develop a strategy for the diagnosis of (inherited) disorders in GC sensitivity. This should also include techniques using materials from patients in whom current GC therapy cannot be interrupted, as well as opportunities to study cells more intensively, without the need for freshly isolated cells.

PATIENTS AND METHODS

Patients

Patients 1–5 have been reported previously. In summary, patients were diagnosed with compensated CR characterized by increased cortisol secretion without Cushingoid features. They showed insufficient suppression of cortisol in a 1-mg dexamethasone (DEX) suppression test. Patient 1 presented with hypertension and oligospermia[19], and his CR was attributed to a heterozygous I559N mutation. The clinical symptoms of patient 2 were hypertension and hypokalemia[17, 19], caused by a heterozygous D641V mutation. Patient 3 presented with symptoms of hyperandrogenism, attributed to a 4-bp deletion ($\triangle_4$) identified at the 3′ boundary of exon 6 and intron 6, removing a donor splice site in one allele, resulting in the transcription of unstable mRNA, consequently decreasing the amount of GRs by 50%[18]. Patient 4 presented with hirsutism and menstrual irregularities, and patient 5 also developed acne, fatigue, and mood disorders, but no GR gene alterations were found explaining the clinical and biochemical CR in these two patients[16].

Patient 6, a 36-yr-old female patient, presented with fatigue, hypertension (systolic blood pressure, 225 mm Hg; diastolic blood pressure, 125 mm Hg), and a slight male pattern of baldness, without signs and symptoms of Cushing's syndrome, hirsutism, or menstrual irregularities [height, 172 cm; 0 SD score (SDS); weight, 66 kg].

In two overnight 1-mg DEX suppression tests, early morning cortisol was insufficiently suppressed [360 and 530 nmol/liter; normal range (N), <145 nmol/liter]. Urinary free cortisol [250–340 nmol/24 h (N, 40–200 nmol/24 h)], as well as early morning cortisol [1280 nmol/liter (N, <850 nmol/liter)], was elevated, accompanied by a slightly elevated plasma ACTH of 120 ng/ml (N, 30–100 ng/ml). Cortisol diurnal rhythm was present, albeit at a higher level. Plasma testosterone varied between 6.7 and 8.4 nmol/liter (N, 1–3 nmol/liter); dehydroepiandrosterone sulfate was 37–43 μmol/liter (N, 3–13 μmol/liter). Bone mineral density of the lumbar spine and hip were normal. The clinical presentation of the patient indicated elevated activity of the HPA axis without signs of Cushing's disease and was typical for CR.

Patient 7 developed renal insufficiency at the age of 40 yr after an unexplained glomerulonephritis. He was one of the first patients undergoing a postmortem donor kidney transplant in The Netherlands in 1972 at the age of 43 yr. Despite low immunosuppressive medication (prednisone, 7.5 mg/d; azathioprine, 100 mg/d), his renal function remained normal and is only slightly impaired today (creatine, 202–263 μmol/d). The 33 yr after transplantation were clinically largely uneventful. He has no other specific diseases. Blood pressure is normal.

Because of this extraordinary clinical course, we suspected abnormal cortisol sensitivity. Despite long-term prednisolone medication, which could not be stopped, substantial

serum concentrations of adrenal androgens were detected, which might indicate decreased GC sensitivity of the HPA-axis feedback system.

Patient 8, a 20-yr-old male patient, was diagnosed at birth with congenital adrenal hyperplasia, and the underlying defect in his 21-hydroxylase gene was recently identified (Timmermans, M. A., F. H. de Jong, unpublished results). He was treated with GCs and MCs (final height, 167 cm; –2.4 SDS; weight, 63 kg). After puberty, he was admitted several times for an Addisonian crisis in relation to intermittent infections. He needed exceptionally high doses of GCs to overcome adrenal insufficiency, indicating GC resistance.

Currently, 20 mg of hydrocortisone three times per day (N, 8–15 mg/m^2·d) or 0.5 mg of DEX four times per day are still insufficient to fully normalize serum ACTH, androstenedione, 17-OH-progesterone, and testosterone levels. Serum LH and FSH levels were fully suppressed, whereas serum TSH, free T_3, and free T_4 were normal. He is also treated with 0.625 mg of 9α-fludrocortisone three times per day (N, 0.05–0.2 mg/d) to reach a normal blood pressure (systolic, 120 mm Hg; diastolic, 70 mm Hg), without orthostasis or peripheral edema. Recently, bone mineral density of the lumbar spine and hip were found to be within normal values.

Patient 9, a 13-yr-old patient, presented with progressive obesity, some nausea, and tiredness. For asthma, she used low-dose inhalation GCs (budesonide 200 μg/d). Growth retardation was noticed (height 142 cm; –3.2 SDS) in combination with general obesity (weight 64.5 kg; +2.8 SDS) and striae. Blood pressure was normal (systolic, 95 mm Hg; diastolic, 63 mm Hg). Serum fasting cortisol level of less than 30 nmol/liter was too low (N, 200–600 nmol/liter) as well as the urinary free cortisol of less than 3 nmol/24 h (N, <500 nmol/24 h). Bone age was 3.5 yr retarded. Bone mineral density of the lumbar spine showed osteopenia (z-score, –2.5 SDS). These clinical features of Cushing's syndrome on low-dose steroid treatment in combination with the suppressed cortisol levels in blood and urine were considered typical for CH.

From all patients, informed consent was obtained, and the Medical Ethics Committee of Erasmus MC, The Netherlands, approved this study.

Whole cell DEX binding, [^{3}H]thymidine incorporation, and mRNA expression of GC-induced leucine-zipper, IL-2, and GR

Blood (70 ml) was drawn into heparinized tubes by venipuncture. Peripheral blood mononuclear leukocytes (PBMLs) were isolated, and the number of GRs per cell (n), their dissociation constant (K_D), and the sensitivity of PBMLs to the inhibition of phytohemagglutinin (PHA)-stimulated incorporation of [^{3}H]thymidine by 100 nM DEX were determined, as described previously[6, 20]. Expression of GC-induced leucine-zipper (GILZ) and IL-2 mRNA levels in response to 100 nM DEX and expression of GR-α, GR-ß, and GR-P splice variants were measured in a real-time quantitative PCR (Q-PCR), as described previously[21, 22].

Epstein-Barr virus transformation of B lymphocytes

Epstein-Barr virus (EBV)-transformed lymphoblast cell lines were established from PBMLs[23, 24]. Cells were grown in RPMI 1640 medium supplemented with 15% fetal calf serum, 100 µg/ml penicillin, and streptomycin at standard culture conditions.

Sequence analysis

The coding sequence of the GR gene including intron/exon boundaries was sequenced in all patients using primers as described previously[25].

RESULTS

Analysis of GR characteristics and expression

Sequence analysis was performed on the nine exons and intronic flanking sequences of the GR in all patients. We have previously reported on the heterozygous mutations in patients 1 (I559N), 2 (D641V), and 3 (△4 bp). In patient 3 also, the earlier reported *N363S* single-nucleotide polymorphism (SNP) was found, enhancing GC sensitivity[26]. In the other patients, several different SNPs were found, but only *ER22/23EK*, heterozygously present in patient 4, has been reported to decrease GC sensitivity[25, 26]. Patient 7 had two heterozygous nucleotide changes in intron 8, 81 bases downstream of exon 8 (G to A) and 9 bases upstream of exon 9 (C to G). Codons 750 and 588 in, respectively, patients 7 and 8 were heterozygously mutated from, respectively, CCC to CCT and CAC to CAT, but this did not cause amino acid changes.

Subsequently, we performed radioligand-binding studies to determine the GR number per cell (n) and the K_D in PBMLs (Fig. 1A) of patients and 14 healthy controls. Patient 3 (△4 bp) showed only half of the normal receptor number per cell, whereas patient 9, suspected of CH, showed an increased receptor number. Only patient 1, carrying the heterozygous I559N mutation, showed decreased affinity for DEX. The data of patients 5 and 7 were not included in Figure 1A because GC medication could not be stopped and subsequently interfered in these binding studies.

Expression of the GR was further analyzed by quantification of mRNA copies of the GR-α, GR-ß, and GR-P splice variants using real-time Q-PCR. Figure 1B shows these levels measured in PBMLs of patients and controls. Patient 3 (△4 bp) shows approximately 50% of the normal amount of GR-α, GR-ß, and GR-P mRNA copies. In patient 9, 70% more GR-α mRNA expression was measured, corresponding with GR number measured in PBMLs in

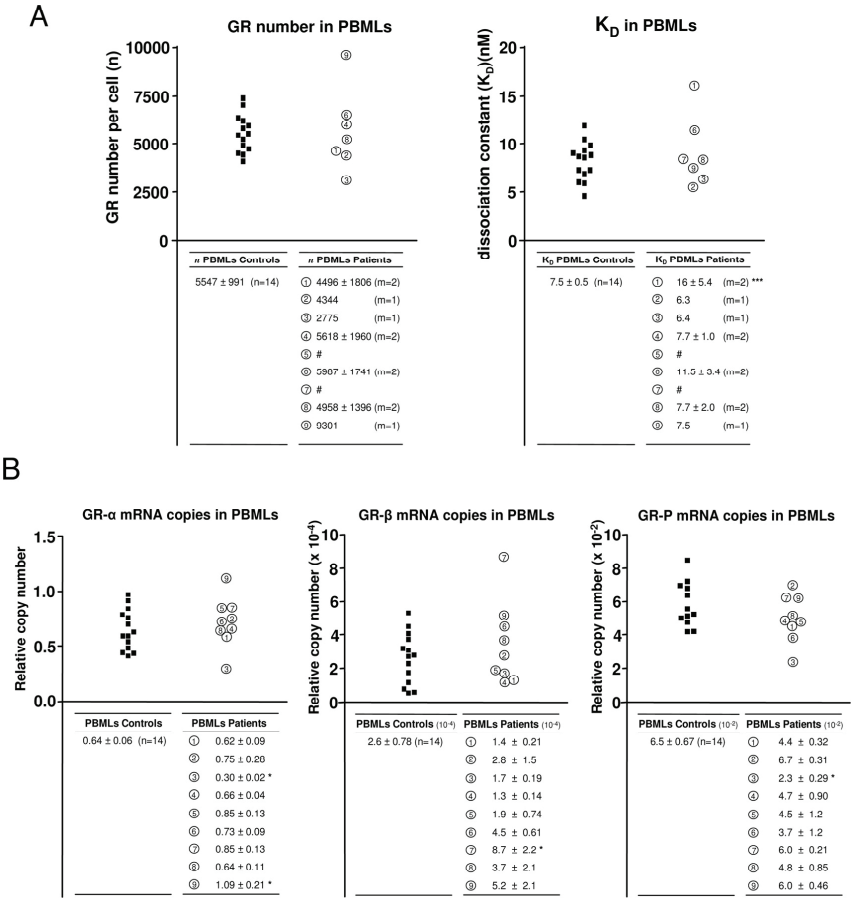

Figure 1. GR characteristics and GR mRNA copy numbers in PBMLs of patients and healthy controls. GR number per cell (n) and KD (A) and relative copy numbers of GR-α, GR-ß, and GR-P splice variants (B) in PBMLs of patients with affected GC sensitivity (circled numbers) and controls (■). From patients 5 and 7, no n or KD could be obtained due to interference of DEX medication (indicated as #). Copy numbers were calculated relative to the levels of the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) by applying the formula 2[CT (HPRT) – CT (GR)]. For further details, see Livak and Schmittgen32. Data represent means ± SEM, and the assay was performed in duplicate with duplicate measurements or as indicated (m). *, $P \leq 0.05$; ***, $P \leq 0.001$ by Student's t test.

the ligand-binding assay. Patient 7 showed 3-fold higher GR-ß expression levels, although no differences for expression of GR-α and GR-P mRNA splice variants were found.

Cellular GC sensitivity

Liganded GR acts together with several cofactor complexes to regulate transcription of GC-responsive genes. Affected GC sensitivity was further investigated by measuring the expression of GILZ and IL-2, two endogenous GC-sensitive genes, which could be strongly up- and down-regulated by GCs, respectively. Figure 2 shows the increase of GILZ (A)

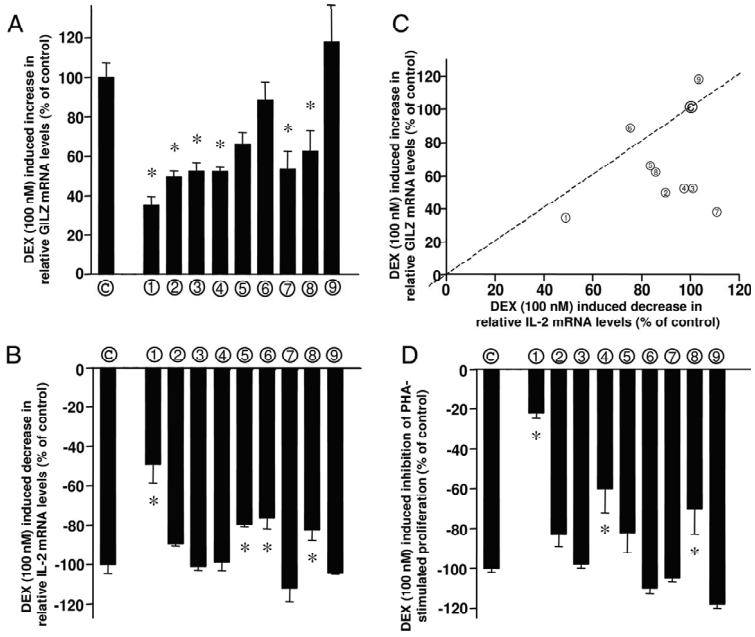

Figure 2. GILZ and IL-2 mRNA expression levels and repression of PHA-stimulated proliferation in PBMLs. Relative increase of GILZ (A) and decrease of IL-2 (B) mRNA levels induced by 100 nM DEX in PBMLs of patients (circled numbers) and controls (©). Cells were incubated for 4 h with PHA and with or without 100 nM DEX, followed by mRNA isolation and quantitation by real-time RT-PCR. Data are presented as the increase of GILZ (A) and decrease of IL-2 (B) mRNA relative to the values in the absence of DEX, which are also presented in C as GILZ vs. IL-2 response. DEX incubations were performed in duplicate, and duplicate real-time Q-PCR was performed for every sample. Levels for controls in arbitrary units were: 100 ± 8 (without DEX) and 1577 ± 115 (100 nM DEX) in the GILZ assay and 100 ± 5 (without DEX) and 28 ± 4 (100 nM DEX) in the IL-2 assay. These response levels were set to 100%. No systematic differences between patients and healthy controls were observed in the absence of DEX (data not shown). For all subjects, PHA treatment in the absence of DEX equally stimulated IL-2 mRNA levels 12- to 18-fold but did not affect GILZ or GR mRNA levels (data not shown). D, Relative inhibition of PHA-induced [3H]thymidine incorporation by 100 nM DEX in PBMLs of patients and controls. The average response in the healthy controls was 100 ± 6 (without DEX) and 18 ± 3 (100 nM DEX) and was set to 100%. Data represent means ± SEM, and the assay was performed in duplicate with incubations in triplicate. The average response in the healthy controls was set to 100%. *, $P < 0.05$ by Student's t test.

and decrease of IL-2 (B) in PBMLs of our patients when stimulated with PHA and 100 nM DEX, relative to levels in the absence of DEX. The average increase/decrease of GILZ/IL-2 mRNA in PBMLs of healthy controls was set to 100%. PHA, necessary to induce IL-2 gene transcription, did not affect expression of GILZ or GR mRNA levels. PHA induction, as well as basal expression levels of GILZ and IL-2 in the absence of DEX, was comparable between patients and controls (data not shown). Patients 2 (D641V), 3 (△4 bp), 4, and 7 showed less up-regulation of GILZ mRNA than the controls (50 ± 3%, 52 ± 5%, 52 ± 3%, and 53 ± 9%, respectively), whereas transrepression of the IL-2 gene was mainly unaffected. In patients 1 (I559N) and 8, transactivation of the GILZ, as well as transrepression of the IL-2 gene, was reduced (GILZ up-regulation and IL-2 repression compared with controls in patient 1, 35 ± 5% and 49 ± 9%, respectively; in patient 8, 62 ± 10% and 85 ± 6%, respectively). The same trend was observed in patients 5 and 6 (in patient 5, 66 ± 6% and 84 ± 5%, respectively; in patient 6, 88 ± 9% and 75 ± 6%, respectively). In patient 9, who overreacted to GC medication, more transcriptional regulation of the GILZ and IL-2 gene seemed to occur, but this was not significantly different from controls. In Figure 2C, the GILZ response is plotted against the IL-2 response. Patients 1, 5, 6, 8, and 9 lie close to the diagonal, indicating defects that equally affect transactivation and transrepression, whereas the marked GILZ defect without substantial alterations in IL-2 response puts patients 2, 3, 4, and 7 off the diagonal in the lower right section, clearly demonstrating that transactivation and transrepression are separable entities.

The PBMLs were also tested in a PHA-stimulated proliferation assay. The decrease in proliferation induced by 100 nM DEX is shown in Figure 2D and is related to the average decrease in the healthy control group set to 100%. Less suppression of proliferation was observed for patients 1 (23 ± 3%), 4 (60 ± 12%), and 8 (70 ± 15%), whereas more suppression was observed for patient 9 (115 ± 2%). Approximately the same trend was shown compared with the results of the IL-2-transrepression assay; only the outcomes for patients 4 and 6 did not correspond. In this proliferation assay, IL-2 gene repression certainly plays a role, but it is carried out over a much longer time scale (4 d, rather than 4 h), and the outcome is formed by the integration of many processes, including apoptosis.

EBV-transformed B-lymphocytes

To obtain permanent cell lines, native B-lymphocytes of patients and controls were transformed with EBV to obtain immortalized lymphoblast cell lines, and then GR number and ligand K_D were measured (Figure 3A). Ligand affinity was not influenced by viral transformation (Spearman's correlation: r = 0.73; *P* = 0.06). Patient 1 (I559N) showed decreased affinity for DEX. GR numbers in lymphoblasts were approximately five times higher than those measured in lymphocytes, even after correction for cell volume (data not shown).

The increase in receptor number after EBV transformation in patients diagnosed as CR, however, was significantly less, whereas the receptor number in the patient who overreacted to GC treatment was significantly higher, than in controls (Figure 3A). Quantifying GR-α and GR-P mRNA expression levels also showed that GR expression is affected after viral transformation (Figure 3B). Figure 4 shows the correlation in lymphoblasts between

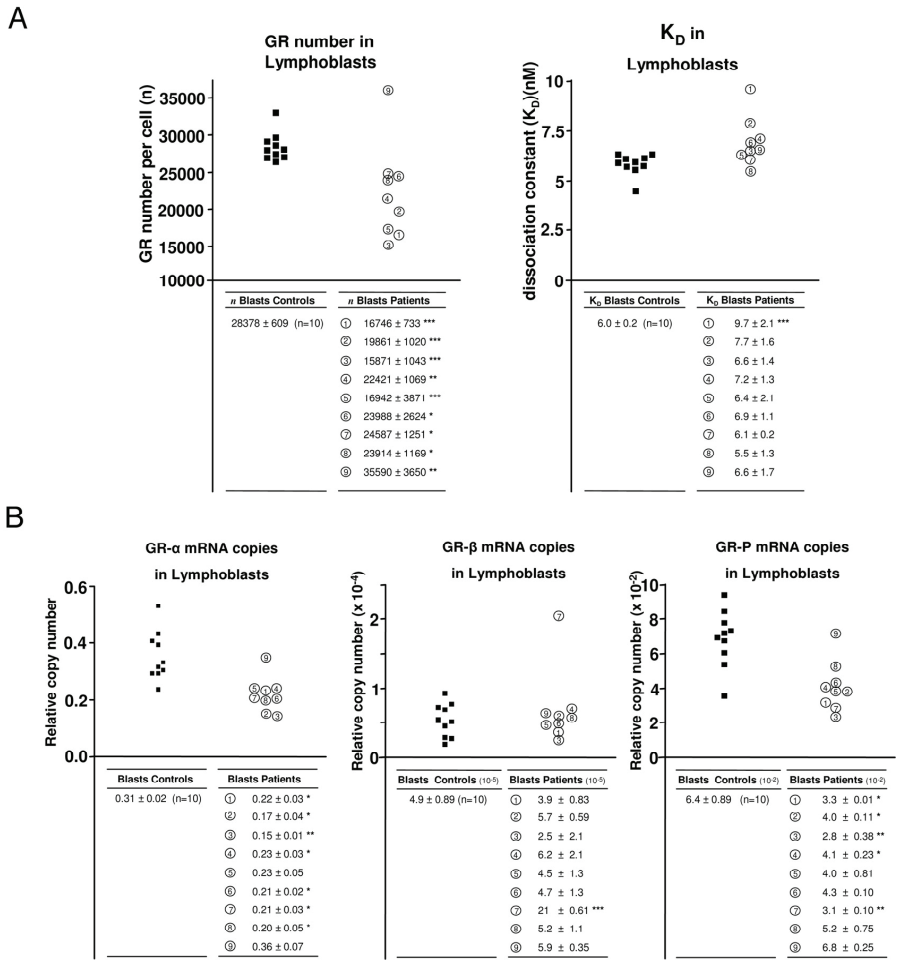

Figure 3. GR characteristics and GR mRNA copy numbers in lymphoblast cell lines of patients and healthy controls. GR number per cell (n) and KD (A) and relative copy numbers of GR-α, GR-ß, and GR-P splice variants (B) in lymphoblasts of patients with affected GC sensitivity (circled numbers) and controls (■). Copy numbers were calculated relative to the levels of the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) by applying the formula 2[CT (HPRT) – CT (GR)]. For further details, see Livak and Schmittgen32. Data represent means ± SEM, and assays were performed at least three times in duplicate, exactly 3 months after transfection. *, P ≤0.05; **, P ≤ 0.01; ***, P ≤0.001 by Student's t test.

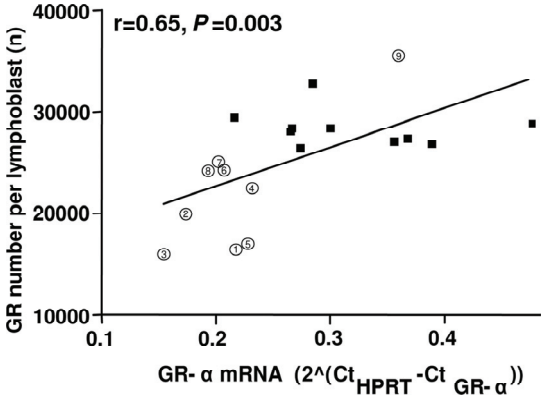

Figure 4. Correlation analysis (Spearman's correlation) between GR number and relative GR-α mRNA copy numbers in lymphoblast cell lines of patients and healthy controls.

GR number determined in the radioligand-binding assay and the number of GR-α mRNA copies, further indicating that GR concentrations in affected patients were different from those measured in controls. GR-ß levels, however, seemed not to be affected by viral transformation, but this might be obscured by the higher variability in quantifying these very low expression levels. Interestingly, GR-ß expression in patient 7 was more than 4-fold higher than in the other subjects, which was also shown, although to a lesser extent, in PBMLs (Figure 1B).

DISCUSSION

We have used three different approaches to study altered GC sensitivity: 1) analysis of the GR characteristics (n and K_D), its coding sequence, and its expression (including mRNA splice variants); 2) examination of GC sensitivity *ex vivo* by measuring responses of endogenous GC sensitive genes (GILZ and IL-2) and the inhibition of mitogen stimulated proliferation; and 3) obtaining permanent cell lines, free of systemic influences, and preceding therapy by transforming B lymphocytes with EBV. A table describing patients, genotype, clinical presentation, biochemical phenotype, and a summary of the data obtained from this study is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

The process through which the I559N and D641V mutations in patients 1 and 2 impair the physiological mechanisms of GC action at the molecular level is multifactorial and involves impaired ligand-binding ability, decreased intrinsic transcriptional activity, and abnormal interaction with certain coactivators[27, 28]. Furthermore, the D641V mutation showed aberrant nucleocytoplasmic trafficking and the I559N mutation exerted a dom-

inant-negative effect on GR-α activity by hampering nuclear translocation[27]. The *ex vivo* results presented in this paper were in line with the results found in these *in vitro* studies[27, 28]: transactivation and transrepression was affected in patient 1. Due to the dominant-negative effect of the GR (I559N), only 30–40% transactivating activity was measured. Transrepression in patient 2, however, was normal, and only decreased transactivational activity was observed. In a previous *in vitro* study, we also observed decreased effects on transactivation of GR (D641V), with no effects on transrepression[28]. An explanation for this discrepancy between patients 1 and 2 may be that transactivation occurs through a mechanism characterized by GR interaction with specific DNA sequences, the GC response elements (GREs), whereas transrepression involves interaction of GR with other transcription factors in the absence of specific DNA binding[29].

Patient 3, expressing only half of the normal number of GRs, might demonstrate the strong relationship between GR number and GC sensitivity. Transactivation is 50% reduced (Figure 2A), whereas maximal transrepression was unaffected (Figure 2, B and C). This might indicate that GR action through transactivation might be more GR-concentration dependent than through transrepression. Malchoff and Malchoff[30] already speculated that alterations of the promoter region or factors modulating gene expression, leading to fewer GRs, could cause CR.

Patients 4, 5, and 6 had hypercortisolism without Cushingoid features, insufficient suppression of early morning serum cortisol concentrations in reaction to 1-mg DEX, variable degrees of androgen overproduction, and fatigue. Cellular sensitivity at the level of transactivation of GILZ was significantly reduced in patients 4 and 5 (Figure 2) and on transrepression of IL-2 also in patients 5 and 6 (Figure 2). GR expression levels and characteristics were normal (Figures 1 and 3), suggesting that the condition of the patients was not caused by reduced GR expression, as might be the result of mutations in the promoter region of the GR gene. However, GR mRNA copy numbers and DEX binding after EBV transformation was lower, which might suggest a defect in GR synthesis or regulation that only becomes apparent in these lymphoblasts. Possible pathophysiological bases of CR in these patients could also be formed by alterations in cellular trafficking or in interactions with other nuclear cofactors. The *ER22/23EK* SNP in patient 4 is reported to slightly decrease GC sensitivity[26] but could not be exclusively responsible for the severe reduced sensitivity as described in this study. At present, hyperactivity of the HPA axis was normalized by low doses of DEX.

Patient 7, who only needed low immunosuppressive medication for a postmortem donor kidney transplant, was suspected of increased immunosuppressive function of the HPA axis. Sequence analysis of the GR gene revealed (among other mutations) a heterozygous C to G mutation in the pyrimidine tract of the exon 9α splice acceptor. Splice site analysis (https://splice.cmh.edu) predicted that the strength of the acceptor splice site is slightly weakened, possibly resulting in skipping of exon 9α in favor of exon 9ß. However,

this is not an absolute effect, because at the level of the mRNA, another heterozygous mutation in this patient (P750P in exon 9α) was also found to be present. But quantitative RT-PCR did show that the GR-ß expression was three to four times higher than in controls, both in PBMLs and in lymphoblasts (Figures 1B and 3B). In the GILZ assay, cells from this patient showed a significantly reduced response, indicating reduced transactivating capacity, whereas in the IL-2 assay, the response was similar to that measured in controls. Our hypothesis is that in this patient, the reduction of transactivating capacity, possibly due to increased expression of GR-ß, results in CR at the level of GRE-mediated GR action (also involved in the feedback sensitivity of the HPA axis), whereas the immunosuppressive function (not GRE mediated) is not affected. As a result, the immune system is exposed to higher compensatory cortisol concentrations and is subsequently relatively suppressed. Increased GR-ß levels have frequently been associated with acquired GC resistance in various disease states (*e.g.* asthma, rheumatoid arthritis); however, increased GR-ß levels in this particular patient may have resulted in positive effects.

In patient 8, transactivation and transrepression activities were decreased (Figure 2), whereas GR characteristics were normal (Figure 1). Extraordinarily high doses of both GCs and MCs were needed to overcome his 21-hydroxylase deficiency. However, this was still insufficient to fully compensate and normalize adrenal function because 17-OH-progesterone, androstenedione, testosterone, and ACTH plasma levels remained elevated. Cofactors influencing both the GR and the MC receptor (MR) activity could be involved, but then, androgen and thyroid receptor function might also be impaired because many coactivators are involved in the functioning of more than one nuclear receptor. Plasma TSH and T_3 were within the reference range, indicating normal thyroid function. Furthermore, plasma LH and FSH were fully suppressed by the elevated testosterone, indicating that androgen receptor function is not impaired either. It is not clear whether cofactors exist that specifically interact with the GR and MR, without influencing other nuclear receptors. Recently, differences between splice variants of steroid receptor coactivator-1 have been reported that strongly interact with GRs and MRs in a promoter-, receptor-, and ligand-dependent way[31]. Disturbances in splicing regulation or tissue-specific expression of these cofactors could have dramatic influences on the cellular GC sensitivity, whereas other nuclear receptor activities might hardly be affected.

The increased sensitivity in patient 9 is mainly due to an increased receptor number (Figures 1 and 3) and slightly increasing cellular sensitivity (Figure 2). Cofactors inducing GR expression or alterations in the promoter region of the GR could be responsible for this. After stopping steroid medication that was used to treat her asthma, the Cushingoid features disappeared.

Viral transformation had no influence on GR quality because similar receptor affinities (K_D) were found for native and transformed cells (Figures 1 and 3) but increased GR number 5-fold. Interestingly, in lymphoblasts of patients diagnosed as CR, induction

was less, whereas in the patient diagnosed as CH, a higher GR number than in controls was measured (Figure 3). Tomita *et al.*[24] already reported that CR patients (from the D641V kindred) showed diminished induction during viral transformation. The molecular mechanism explaining this phenomenon, however, is still unknown. We hypothesize that during viral transformation, autoregulation of the GR occurs that might be impaired or enhanced by CR or CH, respectively. Although this phenomenon limits our possibilities to study the GR in its signaling context, due to noncomparable GR concentrations, the abnormalities in GR up-regulation in lymphoblasts of patients might be an additional indicator of altered GC sensitivity. Plotting GR number per lymphoblast against GR mRNA copy number (Figure 4) grouped all patients diagnosed as CR into the lower left sector, regardless of the molecular basis of the defect. Compared with measuring GR characteristics and GC response in freshly prepared cells, EBV transformation is more laborious. However, measuring GR up-regulation during EBV transformation seemed to be the most powerful tool to differentiate CR and CH from controls. The other markers (GILZ, IL-2, and proliferation) are easier to obtain, but as individual markers, they are less powerful because they sample distinct aspects of GC sensitivity (transactivating, transrepressing capacity but also proliferation processes, including apoptosis), which can differ strongly between patients.

In conclusion, for the appropriate diagnosis of CR or CH, a careful interpretation of clinical presentation is essential, but it is subsequently also important to quantify these syndromes biochemically. To do this, we have investigated GR characteristics and GC response using freshly isolated PBMLs and permanent cell lines. The results of these approaches are illustrated in this study by describing nine patients with suspected abnormalities in GC sensitivity, all with a unique clinical course and background.

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Chapter 6

Ficol separated mononuclear cells from sepsis patients are contaminated with granulocytes

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ABSTRACT

Objective:
To determine the cell content and purity of Ficol separated peripheral blood mononuclear cells and granulocyte isolates in sepsis patients compared to healthy controls.

Design and Setting:
Prospective study in the adult and pediatric intensive care departments of the Erasmus University Medical Center in the Netherlands.

Patients:
Three sepsis patients (2 adults, 1 child) and 4 healthy controls.

Measurements and results:
Blood leukocytes were separated by Ficol in an interface and a bottom fraction. The cell content and purity was analyzed by cytospin and flow cytometric immuno-fluorescence. In sepsis patients, the interface consisted of 11-52% mononuclear cells only, due to high contamination with granulocytes (48-89%). This was in contrast to a high percentage of mononuclear cells (88-100%) in healthy controls. The bottom fraction showed a cell purity of ≥92% polymorphonuclear granulocytes in sepsis patients as well as in healthy controls.

Conclusions:
Ficol-separated leukocytes of sepsis patients are not suitable for studying mononuclear cells but can be used for studying granulocytes with high purity. The mononuclear cell fraction is highly contaminated with granulocytes. Additional separation techniques are necessary to obtain a pure cell fraction.

Key words: sepsis, Ficol, mononuclear cells, granulocytes.

INTRODUCTION

Leukocytes play a critical role in the regulation of infectious diseases. Studying the changing characteristics of leukocytes during infectious disease is important for a better understanding of its pathogenesis [1]. In ex vivo studies, the Ficol separation technique is frequently used for isolation of mononuclear cells from human peripheral blood leukocytes. The standard procedure of this Ficol technique was described in 1976. Blood leukocytes, layered over a Ficol solution, show differential migration during centrifugation, resulting in the formation of layers containing different cell types. Because of their lower density, the peripheral blood mononuclear cells (lymphocytes and monocytes) are found at the interface between the plasma and the Ficol, while granulocytes and erythrocytes reside in the bottom fraction. An advantage of this technique is the relatively high cell purity in the two fractions in healthy controls (>98%) [2].
To date, Ficol separation is increasingly being used to study mononuclear cells in different clinical research settings like studying sepsis patients. Differences in mononuclear cell characteristics, such as expression of inflammatory proteins, of sepsis patients are often compared to controls [1, 3-8]. However, no data are available on cell purity of the Ficol separated cell fractions in sepsis patients.
Our aim was to determine the cell content and purity of the Ficol separated mononuclear cell and granulocyte isolates in sepsis patients compared to healthy controls.

MATERIALS & METHODS

Subjects

Peripheral blood was obtained from 3 sepsis patients and from 4 healthy controls. Two adult patients and one child, admitted to the intensive care unit, were studied within 24 hours after diagnosed with sepsis as defined by the international consensus [9, 10]. Patients with neutropenia or immunosuppressive therapies were excluded. The APACHE-II [11] (adults) and pediatric risk of mortality score (PRISM) [12] (child) were used as measure of severity of illness. The healthy controls, aged 51,36,21 and 3 years, did not suffer from any acute or chronic illness. The Medical Ethics Committee approved the protocol. Informed consent was obtained from the patients or the parents.

Leukocyte isolation

Blood (5mL) was drawn into heparinized tubes (diameter 10 mm) at 9:00 a.m and processed immediately. Blood was diluted 2-fold with saline and layered over a 10 mL Ficol-

Paque-Plus solution (density 1.077/mL, Amersham Biosciences, Uppsala, Sweden). Density gradient centrifugation was performed at 400 x g for 20 min at 20°C. The interface was isolated and after centrifugation (850 x g for 5 min) washed twice in saline. From the bottom fraction, after lysing the erythrocytes using 10 mL of lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH7.35 for 30 min on ice), cells were isolated by centrifugation (850 x g for 5 min) and washed twice in saline. The final cell pellets were suspended in saline. Viability, tested by trypan blue, was more than 95%.

Cytospin and flowcytometry

The leukocytes from the Ficol-separated cell fractions were analyzed by both cytospin and by flow cytometric immuno-fluorescence (FACS). Cytospin samples were prepared with a cell suspension of 1000 cells in 100 µL of saline. Centrifugation was performed in a Rotofix32(Hettich,Tuttlingen,Germany) for 5 minutes,72 x g. Preparations were stained with May-Grünwald-Giemsa staining [13]. Leukocyte differentiation was performed using a microscope (Axioskop20,Zeiss,Germany)
Ficol separated samples were analyzed with FACS for cell subsets. Cell types were analyzed by forward scatter and side scatter to identify size and granulation of the cells. Immuno-fluorescent labels used monoclonal antibodies (mAb) directly conjugated to fluorescein(FITC) or phycoerythrin(PE). Anti-CD14 antibodies were used as a monocyte marker. CD14-antigens are not expressed on lymphocytes and granulocytes. Anti-CD45 antibodies were used as a marker for lymphocytes (high expression) and granulocytes (low expression). Twenty µL of the dual mAb combination CD45-FITC/CD14-PE (BectonDickinson, SanJose,CA) was added to 100µL of the sample. After 30min of incubation at room temperature, FACS lysis buffer (BD) was added for lysing the remaining erythrocytes. After washing, cells were analysed on flowcytometer (FACScan,BD) using SimulSet software (BD) for data analysis. A total of 1000 to 2000 gated leukocyte events, were recorded from each tube. Information from forward scatter, side scatter, CD14 and CD45 staining were integrated to analyze the percentages of cell subsets. Comparison of mean percentages of granulocytes in controls compared to sepsis patients was performed by one-way ANOVA analysis. A p-value of <0.05 was indicated as significant.

RESULTS

Patients (Table 1)

Patient 1, a 71-year-old male, was diagnosed with urothelial cell carcinoma. A radical cystoprostatectomia was performed, complicated by pulmonary embolism and a per-

Table 1. Patient characteristics

	patient 1	patient 2	patient 3	reference
Gender	male	male	male	values
Age	71	77	7	
APACHE-II[a]/PRISM[b] score	18	13	2	
C-reactive protein (mg/L)	220	248	84	<10
Leukocytes (x109/L)	78	13.4	20	3.5-10
Differentiation:				
Lymphocytes (%)	3	6	5	15-50
Monocytes (%)	8	2	4	3-10
PMN granulocytes (%)	76	56	69	40-80
Myelocytes (%)	6	12	0	0
Band cells (%)	7	24	22	0

[a]patient1 and patient2; [b]patient3. PMN=polymorphonuclear

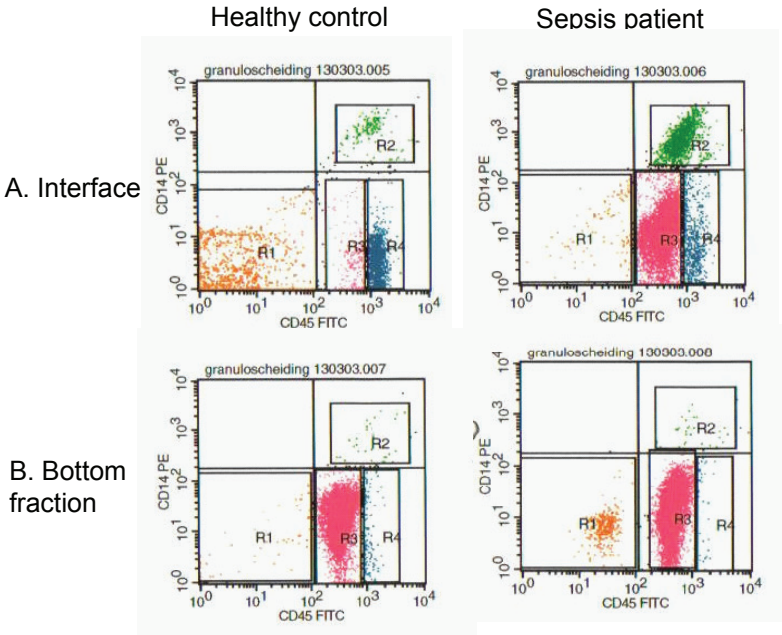

Figure 1. FACS analysis of cells from a healthy control (cytospin: 92% mononuclear cells and 8% neutrophils) compared to those of sepsis patient 1 (table 1). Figures from patient 2 and 3 were similar. Ficol-separated leukocytes from the interface (a) and bottom fraction (b) were stained with anti-CD14 and anti-CD45 antibodies. Orange=CD45 negative cells like trombocytes or erythrocytes (R1), green = monocytes (R2), pink = granulocytes (R3), blue=lymphocytes (R4).

forated peptic ulcer. Five weeks after admission, sepsis with multi-organ failure was diagnosed. He had an APACHE-II score of 18 points. His leukocyte count was severely

elevated with immature granulocytes in the differentiation. No hematological disease was found. Blood culture was negative, urine and wound culture contained a Klebsiella species.

Patient 2, a 77-year-old male, was diagnosed with a perforated peptic ulcer, due to use of nonsteroidal anti-inflammatory drugs, hypovolemic shock and respiratory insufficiency. Ten days after a gastric resection, relaparotomy was performed for colonic perforation. One day later, sepsis was diagnosed. His APACHE-II score was 13 points, leukocyte count showed a reduced lymphocyte count and a shift towards immature granulocytes. Blood-culture remained negative, wound and urine culture contained pseudomonas aeruginosa. For both patients, therapy was instituted with mechanical ventilation, vasopressors, sedatives and antibiotics. Both patients died of complications related to the sepsis.

Patient 3, was a 7 year old boy who became ill one day before admission to the pediatric intensive care. Clinical symptoms were fever (temperature39.8 °C), headache, vomiting and petechiae. On admission the boy was disoriented and showed clinical signs of meningococcal sepsis. His pediatric risk of mortality score was low (2points). He was treated with intravenous antibiotics and dexamethasone. Liquor culture was not performed and blood culture remained negative. He recovered steadily in a week.

Both cytospin and FACS analysis showed similar results in cell analysis. In sepsis patients, the interface, contained mononuclear cells in a range of 11-52% and granulocytes in a range of 48-89%(Fig1a+2a). The absolute numbers of granulocytes (Fig2, white bar) for controls, patient 1,2 and 3 were: 0.8, 91, 24 and 8 million respectively. In healthy controls, the interface contained between 88-100% of mononuclear cells and granulocytes in a range of 0-12%(Fig1a+2a). Mean percentage of granulocytes in the interface of healthy controls (11%), was significantly lower compared to sepsis patients (62%,p-value=0.007).

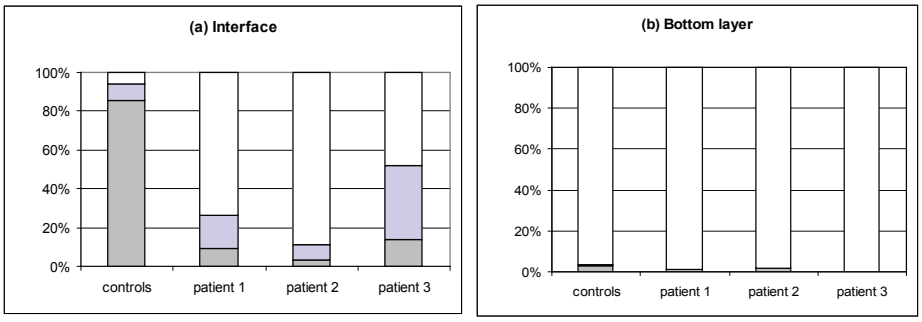

Figure 2. These results correspond to the percentages of cell types in the interface (a) and bottom fraction (b) from FACS analysis of Ficol separated leukocytes of 4 healthy controls (mean percentage) and 3 sepsis patients. Gray bar = lymphocytes, dotted gray bar=monocytes, white bar = granulocytes.

The FACS cell type analysis of the bottom fraction from sepsis patients consisted of granulocytes in a range of 97-99.8% (Fig1b+2b). In healthy controls this fraction showed a range of 92-98% granulocytes (Fig1b+2b). No significant difference was found in mean percentage of granulocytes in the bottom fraction of healthy controls (91%) compared to sepsis patients (91%,p-value=0.97).
Cytospin analysis showed that the granulocytes from sepsis patients from both top fraction and bottom layer consisted of immature forms (4-21% band cells and myelocytes) as well as polymorphonuclear granulocytes. In healthy controls, no immature forms but only polymorphonuclear granulocytes were found (data not shown).

DISCUSSION

Our study demonstrates that leukocyte isolates from a Ficol-separated interface of sepsis patients contains low percentages of mononuclear cells due to high contamination with granulocytes (Fig1+2a) compared to healthy controls. This is the result of a reduction in absolute number of lymphocytes during sepsis and an increase of migration of granulocytes to the interface. Cytospin differentiations showed that these granulocytes consisted of immature forms (band cells and myelocytes) and polymorphonuclear granulocytes.
Immature granulocytes are normally not present in the peripheral blood of healthy individuals [1]. However, in sepsis patients, they are frequently observed due to the induced stress response. Because of their different size and density compared to polymorphonuclear granulocytes, their migration through the Ficol is also different [2]. In addition, we hypothesize that standard separation techniques might not suffice for leukocytes during sepsis because immune activation changes them into a more immature and adhesive cell type and thus may interfere with standard separation techniques.
In the first description of Ficol separation on human blood of healthy donors, mononuclear and granulocyte cell isolates with high purity were found[2]. In pigs with classical swine fever, contamination of the mononuclear cell fraction with immature granulocytes after Ficol separation was reported[14]. In humans, several reports from burn patients mention contamination of the Ficol mononuclear cell preparations with nonlymphoid cell populations[15-17]. In sepsis patients, a number of studies have been performed using Ficol separation for studying mononuclear cells. However no data are available on cell content or purity in these cell isolates. Interpretation of studies, using Ficol separated mononuclear cells of sepsis patients, is difficult without identification or purification of cell types. Additional separation techniques are necessary to obtain purified cell populations of sepsis patients. Usage of (immuno) magnetic beads[18] could be an option. As CD16 and CD11b expression appear during maturation of granulocytes, these antibodies can be considered for further separation[19]. In addition usage of cell preparation tubes with pre-

filled Ficol can be considered to reduce handling. Future studies with larger numbers and age-matched controls are warranted to confirm our findings.
In conclusion, this study demonstrates that in sepsis patients, the Ficol-separation technique is not suitable for studying mononuclear cells, but can be used for studying granulocytes with high purity. For the mononuclear cells, additional separation techniques are warranted. Cell content and purity should be checked.

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Chapter 7

Glucocorticoid receptor mRNA is decreased in neutrophils of children with sepsis.

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Submitted

ABSTRACT

Objective:
Corticosteroids are used in sepsis treatment to benefit outcome. However discussion remains on which patients will benefit from treatment. Inter-individual variations in cortisol sensitivity, mediated through the glucocorticoid receptor, might play a role in the observed differences. Little is known on endogenous changes of the glucocorticoid receptor levels during sepsis. Our aim was to study changes in mRNA levels of three glucocorticoid receptor splice variants in neutrophils of children with sepsis.

Patients and Design:
Twenty-three children, admitted to the pediatric intensive care unit with sepsis or septic shock were included. Neutrophils were isolated from peripheral blood at day 0, 3, 7 and after recovery (> 3months). mRNA levels of the glucocorticoid receptor splice variants GR-α, GR-P and GR-β were measured using quantitative real-time polymerase chain reaction.

Main results:
Neutrophils from sepsis patients showed decreased levels of glucocorticoid receptor mRNA of the GR-α and GR-P splice variants on day 0 compared to after recovery. GR-α and GR-P mRNA levels showed a gradual recovery on days 3 and 7 and normalized after recovery (p-value for GR-α = 0.000001 and for GR-P= 0.0001). GR-β mRNA levels did not change significantly during sepsis (p 0.3). No association was found between day 0 glucocorticoid receptor levels and gender, presence of shock, diagnosis, pediatric risk of mortality score, serum levels of C - reactive protein, cortisol, ACTH, or medication.

Conclusions:
Children with sepsis or septic shock showed a transient depression of glucocorticoid receptor mRNA in their neutrophils. This feature may represent a tissue specific adaptation during sepsis leading to increased cortisol resistance of neutrophils. This study adds to understanding the mechanism of cortisol sensitivity in immune cells. Future treatment strategies, aiming at timing and tissue specific regulation of glucocorticoids, might benefit patients with sepsis or septic shock.

INTRODUCTION

Sepsis is a systemic response to a severe infectious disease with still a high mortality and morbidity despite improving treatments. Cortisol has an important role in counterbalancing the immune activation to infection. An adequate cortisol stress response is essential for sepsis survival [1], but treatment with high doses of corticosteroids did not improve outcome[2], Low-dose corticosteroid replacement in adult patients with septic shock has been shown to have a beneficial effect on outcome in adults[2]. Discussion on which patient will benefit from corticosteroid treatment is still ongoing[3, 4].

Inter-individual variations in the endogenous cortisol response to stress might play a role in the explanation of the observed differences[5, 6]. At a tissue level, the cortisol effect is determined by the glucocorticoid receptor[7-10]. Three different 3'-splice variants of the glucocorticoid receptor have been reported: GR-α, the most abundant, binds ligand and is functionally active; GR-P, is thought to enhance the function of GR-α[11]; and GR-β, is a dominant negative inhibitor of GR-α action [10, 12]. Changes in levels of the different splice variants is thought to regulate glucocorticoid sensitivity (i.e. the response to cortisol) in a tissue specific way[13, 14]. In lymphocytes of sepsis patients, increased cortisol sensitivity (measured by a thymidine incorporation assay in dexamethasone stimulated lymphocytes), has been found compared to controls[15]. Neutrophils play an important role in the defense against bacterial infections. Neutrophils of healthy persons are known for their low glucocorticoid receptor expression[16] and their reduced cortisol sensitivity compared to other immune cells[8]. Activation of neutrophils, through infection, leads to a pro-inflammatory state demonstrated by reduced apoptosis, increased adherence to the endothelium, extravasation, phagocytosis and production of pro-inflammatory cytokines during sepsis[17]. This leads to a better microbial clearance, which is beneficial for combating sepsis in the acute phase[17]. However, it also leads to tissue damage in prolonged sepsis[17], contributing to multiple organ failure. Regulation of neutrophil activity by changing its cortisol sensitivity during sepsis could therefore enhance pathogen elimination or diminish organ failure. Neutrophils and their possible changes in cortisol sensitivity during sepsis have not been studied before at the level of the glucocorticoid receptor.

We hypothesized that neutrophils during sepsis would temporarily become more resistant to the anti-inflammatory effects of cortisol by transient down-regulation of their glucocorticoid receptor mRNA levels. Therefore, we aimed to study changes in glucocorticoid receptor mRNA levels in neutrophils of children with sepsis longitudinally.

MATERIALS & METHODS

Patients

Patients diagnosed with sepsis were studied within 24 hours after diagnosis. All children were admitted to the pediatric intensive care unit. Sepsis and septic shock were defined in accordance to the criteria set forth by the International Pediatric Sepsis Consensus Conference[18]. Shock was defined as persistent hypotension or evidence of poor end-organ perfusion[18]. Patients with neutropenia or immuno-suppresive therapies were excluded. The pediatric risk of mortality (PRISM) score was used to determine the severity of illness of individual patients[19]. A higher score means a higher risk of mortality. Blood samples were taken immediately after admission and informed consent, on day 0 (t=0), and at 9:00 a.m. on day 3 (t=3), day 7 (t=7) and after recovery (> 3months).
For comparison, blood was obtained from 20 healthy controls of the laboratory staff aged 21 – 58 years, who did not suffer from any acute or chronic illness. Using healthy children as controls, was practically impossible and deemed unethical because a fairly large volume of blood is needed, it is obtained by venapuncture and it should be obtained instantly when a sepsis patient is admitted.
The protocol was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam and parents gave their written informed consent to the study.

Neutrophil isolation

All procedures for RNA isolation were performed immediately on fresh blood in all patients. Blood (5 ml) was drawn from an arterial catheter or venapuncture into heparinized tubes. Neutrophils were isolated by the Ficoll separation technique as describes before.[20] Viability was tested by trypan blue and more than 95% of cells were viable. Purity of cell type subpopulation was analysed by cytospin and flow cytometric immuno-fluorescence analysis and was shown to be of high purity (>98%).[20]

RNA isolation, RT-reaction, and Quantitative real time PCR

For RNA isolation, a cell suspension of 8 x 10^6 neutrophils in 200 µl saline was dissolved in 1 ml of trizol reagent (Invitrogen, Breda, The Netherlands) and incubated for 5 minutes at room temperature. After adding 0.2 ml chloroform, tubes were shaken vigorously for 15" and incubated for 3 minutes at room temperature. After 10' centrifugation at 4 °C and 8600 x g, the RNA, now in the aqueous phase, was transferred to a new tube, precipitated with 0.5 ml isopropanol, incubated for 10' at room temperature and centrifuged

for 10 minutes at 4 °C and 8600 x g. The supernatant was washed with 1 ml 70% ethanol, vortexed and centrifuged for 5 minutes, 4 °C at 4300 x g. The remaining RNA pellet was dissolved in 30 µl RNAse-free water and incubated for 10 minutes at 55 °C. RNA concentrations were measured using a spectrophotometer (Nanodrop, Los Angeles U.S.A.). The RNA sample was stored at -80 °C. cDNA was synthesized from 200 ng RNA in a total volume of 50 µl, using a reverse transcription (RT)-reaction as described previously[21]. Quantitative real time PCR was performed for GR-α, GR-P and GR-β splice variants. Correction for assay variability was performed using the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) of which expression levels are stable and not influenced by glucocorticoids (not shown). The primer sequences and reaction mix used have been previously described[21]. The reaction contained 2 µl cDNA template (corresponding to 8 ng total RNA in the RT-PCR), 2.5 µl reaction buffer, 2.5 µl MgCl2, 1 µl dNTP's, 0.125 µl polymerase, 0.3 pmol/µl forward and reverse primer (0.5 pmol/µl for HPRT) and 0.2 pmol/µl probe, adding water to a total volume of 25 µl. The reactions were carried out in an ABI 7700 Sequence Detector System (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). After initial heating at 95° C for 8 minutes, samples were subjected to 40 cycles of denaturation at 92 °C for 15 seconds and annealing and synthesis for 1 minute at 60 °C. mRNA levels of the GR-α, GR-P, GR-β splice variants and HPRT were calculated using the comparative CT Method, according to the manufacturer's guidelines.

Statistical analysis

Data were analyzed using SPSS for windows, release 12.0.1 (SPSS, Chicago, IL, USA). For analysis of mean splice variant expression, a paired samples t-test was used. The time course of splice variant mRNA levels at 4 different time points was analyzed using mixed model analysis, which allows the use of incomplete data in follow-up. Correlations between splice variant mRNA levels and continuous variables were analyzed using Pearson's correlation coefficient. For analysis of differences in splice variant levels between sexes, presence of shock, diagnosis or medication, the independent samples t-test was used. A p-value <0.05 was considered significant.

RESULTS

Twenty-three children with sepsis or septic shock were included in the study (Table 1), of whom 13 (57%) were male. Median age was 2.7 years ranging from 3 months to 14 years. The most frequent diagnosis was meningococcal sepsis in 15 (65%) patients. Fourteen (61%) were diagnosed with shock. Sixteen (70%) needed mechanical ventilation. Median admission duration on the intensive care unit was 2.0 days (range 1-16). Median

Table 1. Patient characteristics

nr	gender	age (years)	diagnosis	Ventilated	ICU length of stay (days)	mort	GC	PRISM score normal=0	cortisol (nmol/L) normal<750	ACTH (pmol/L) normal<40	CRP mg/L normal<10
1	F	1.2	meningococcal sepsis	no	1	surv	no	7	848		365
2	F	2.3	meningococcal sepsis	no	1	surv	no	13	1,349	3.5	84
3	M	2.7	meningococcal sepsis	no	1	surv	no	11	638		65
4	F	2.9	meningococcal sepsis	yes	1	surv	yes	17	1,645		156
5	F	5.8	meningococcal sepsis	yes	2	surv	no	11	1,006	4.8	229
6	M	2.4	meningococcal sepsis	no	2	surv	yes	19			201
7	M	2.1	meningococcal sepsis	no	2	surv	no	5	619	2.0	89
8	M	3.0	meningococcal sepsis+shock	yes	15	surv	no	15	696	238.0	85
9	F	4.0	meningococcal sepsis+shock	yes	7	surv	no	37	782	141.0	25
10	M	0.3	meningococcal sepsis+shock	yes	13	surv	no	14	995	39.3	150
11	F	1.7	meningococcal sepsis+shock	yes	2	surv	no	23	601	5.2	104
12	M	5.1	meningococcal sepsis+shock	yes	4	surv	no	19	1,047	13.6	51
13	M	1.4	meningococcal sepsis+shock	yes	5	surv	no	31	244	179.0	78
14	M	1.8	meningococcal sepsis+shock	yes	1	surv	yes	14	394	2.4	176
15	M	2.1	meningococcal sepsis+shock	yes	2	surv	yes	30	2,901	1.9	408
16	M	2.8	sepsis+ shock eci	yes	1	surv	yes	20	531	6.4	364
17	M	12.6	sepsis eci	no	0	surv	no	3	792	1.0	91
18	M	7.7	sepsis eci	no	0	surv	yes	1	1,162	9.5	46
19	F	14.6	toxic shock syndrome	yes	3	surv	no	21	3,167	1.0	112
20	F	3.3	toxic shock syndrome	yes	13	surv	no	20	525	1.3	210
21	F	0.7	pneumococcal sepsis+shock	yes	16	surv	yes	29	1,241	3.2	346
22	F	2.4	pneumococcal sepsis+shock	yes	16	surv	no	38	2,344	141.0	53
23	M	14.8	staphylococcal sepsis+shock	yes	1	dead	no	29	1,948	4.1	323

F=female, M=male, mort=mortality, GC= glucocorticoid therapy, surv=survived

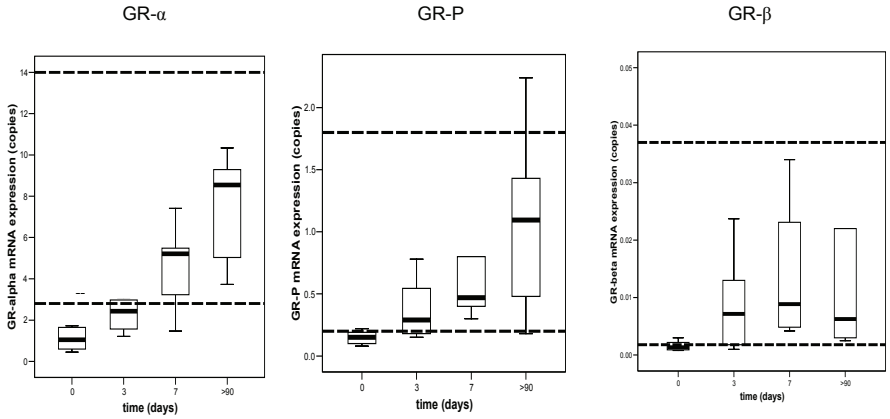

Figure 1. Levels of glucocorticoid receptor splice variants GR-α, GR-P and GR-β mRNA in neutrophils of children with sepsis, measured at day 0, 3, 7 and after recovery. Y-axis represents mRNA levels (copies) measured by real-time polymerase chain reaction. The dotted reference lines represent the ± 2 standard deviations of healthy controls.

PRISM score was 19 points (range 1-38). Glucocorticoid treatment was used in 7 patients, etomidate in 5, midazolam in 13 and inotropic support in 15 patients (Table3). Median level of C-reactive protein (CRP) at admission was 112 mg/L (range 25-408), cortisol 921 nmol/L (range 244-3167), ACTH 4.8 pg/L (range 25-408). Of the 23 children who were included, one died within 24 hours after diagnosis (patient 23, Table1).

A blood sample was obtained from all 23 patients at day 0, from 11 patients at day 3, from 5 patients at day 7 and from 15 patients after recovery (> 3 months after t=0). The drop-outs on day 3 and 7 were due to prior discharge from the ICU. From 7 patients no recovery sample was obtained: 2 dropped out of the study because the parents refused venapuncture after recovery, 3 were missed during follow up and in 2 patients RNA isolation failed in the laboratory.

Glucocorticoid receptor mRNA levels are shown in Figure 1. Sepsis children showed significantly lower levels of GR-α and GR-P in their neutrophils at t=0 compared to after recovery (p= 0.00004 and 0.0001 respectively, Figure 1). The GR-β levels did not change

Table 2. Correlation of glucocorticoid receptor levels with patient characteristics.

	cc GR-α	p-value	cc GR-P	p-value	cc GR-β	p-value
PRISM	-0.16	0.47	-0.14	0.52	-0.17	0.94
CRP	0.25	0.91	-0.79	0.72	-0.54	0.82
cortisol	0.30	0.18	0.15	0.52	0.15	0.52
ACTH	0.38	0.11	-0.32	0.18	0.17	0.49

GR= glucocorticoid receptor, cc= Pearsons correlation coëfficient, PRISM= pediatric risk of mortality score,. CRP= C-reactive protein.

Table 3. Mean glucocorticoid receptor levels at t=0 grouped by sexe, diagnosis or medication.

		n	GR-α	p-value[a]	GR-P	p-value[a]	GR-β	p-value[a]
Gender	male	13	1.27	0.92	0.18	0.89	0.0012	0.21
	female	10	1.23		0.19		0.0048	
Shock	no	9	1.36	0.67	0.19	0.94	0.0055	0.19
	yes	14	1.19		0.18		0.0014	
Diagnosis: meningococcal	no	8	1.54	0.29	0.24	0.23	0.0013	0.44
	yes	15	1.11		0.15		0.0036	
Glucocorticoids	no	16	1.45	0.14	0.23	0.04	0.0015	0.23
	yes	7	0.82		0.08		0.0052	
Ethomidate	no	18	1.43	0.09	0.21	0.27	0.0031	0.70
	yes	5	0.65		0.11		0.0017	
Midazolam	no	10	1.38	0.59	0.20	0.72	0.0013	0.44
	yes	13	1.17		0.17		0.0036	
Inotropic support	no	8	1.48	0.41	0.2	0.79	0.0011	0.43
	yes	15	1.14		0.18		0.0036	

[a] Comparion of means by independent sample t-test

significantly (p 0.3, Figure 1). Longitudinal analysis showed a linear increase for GR-α and GR-P mRNA (p= 0.000001 and 0.0001, respectively) from t= 0 to 3 and 7 days until recovery. After recovery, mean GR-α, GR-P and GR-β mRNA levels were not different in sepsis patients compared to the levels in 20 healthy controls (p-value = 0.8, 0.9 and 1.0 respectively). The ± 2 standard deviation of the healthy controls is indicated by a dotted line in figure 1.

No correlation was found between splice variant levels at day 0 and PRISM score, serum levels of CRP, cortisol or ACTH (Table 2). No differences in glucocorticoid receptor mRNA levels were found in groups divided by gender, presence of shock, diagnosis or medication (Table 3).

DISCUSSION

This study shows that in neutrophils of children with sepsis or septic shock, glucocorticoid receptor mRNA levels are suppressed (Figure 1). During follow-up, the GR mRNA levels gradually increase at days 3 and 7 and normalize after recovery (Figure 1). Our study demonstrates a transient decline of glucocorticoid receptor mRNA levels in neutrophils during sepsis. Therefore, our results are in line with enhanced cortisol resistance and immune activation of neutrophils during sepsis. Activated neutrophils are important in fighting sepsis and lead to an enhanced innate immune response to bacterial invasion[22]. They produce an anti-infectious response by producing pro-inflammatory mediators like

cytokines and nitric oxide [22, 23]. Glucocorticoids support this process by increasing granulocyte number and activation status[24]. This could be a useful adaptation in the acute phase of sepsis when fighting the bacterial infection has priority. However, in case of prolonged sepsis, as is seen more often in adults compared to children, this change in neutrophil activation status might lead to tissue damage, multiple organ failure and thus not benefit the patient. Future studies are needed in adults and on different time points of disease. Timing of corticosteroid therapy might be important for its effect.

The decrease of GR mRNA levels in sepsis neutrophils could represent a tissue specific effect. Other studies reported glucocorticoid receptor down-regulation as well as up-regulation in different tissues, measured by protein amounts (western blots) or by binding-assays. In endotoxin treated rats, reduced GR binding in liver, lung and spleen was seen [25, 26]. However, increased GR mRNA levels and binding activity were found in muscle [27, 28]. In humans, peripheral blood lymphocytes from sepsis patients showed an increased cortisol sensitivity which suggests increased amount of GR[15]. The currently available technologies provide the possibility of measuring mRNA levels quantitatively. To our knowledge, this is the first time that glucocorticoid receptor mRNA levels are studied in neutrophils during sepsis. The transient decrease in GR mRNA levels in sepsis neutrophils, found in our study, differs from other tissues described in literature. This indicates a tissue specific change in cortisol sensitivity during sepsis. Studies aiming at development of tissue specific corticosteroid therapy might be important for improving future treatment strategies.

The transient change of glucocorticoid receptor mRNA levels in our study reflects the clinical improvement seen in the first week in children with sepsis. The suppression of glucocorticoid receptor mRNA levels also coincides with previously reported elevation in serum cortisol and decrease of serum cholesterol, a cortisol precursor, measured in children with sepsis[5, 29]. The cause of decrease of glucocorticoid receptor mRNA levels is not known. It could have been altered by cytokines. Previous in vitro studies showed that after stimulation of neutrophils with pro-inflammatory cytokines, a lower GR-α / GR-β ratio was found[10, 30, 31]. We have no indication that the mRNA level was influenced by factors like gender, presence of shock, diagnosis, PRISM, plasma levels of cortisol, ACTH, CRP, or use of medication while no associations were found with any of these variables(Table2+3).

Our study results might be limited by sample size. Further, we aimed at studying children with the advantage of studying pure sepsis without interference of chronic disease or medication. However, the results might not apply to adults. We studied neutrophils only; studying other types of immune cells would be interesting but might be hampered by difficulty of isolating pure cell subpopulations. Mechanistic investigation is needed to identify specific regulators of GR expression and splice variants during sepsis.

In conclusion, children with sepsis showed a transient depression of the glucocorticoid receptor splice variants GR-α and GR-P mRNA in their neutrophils. This feature may represent a tissue specific adaptation during sepsis leading to increased cortisol resistance of neutrophils. Understanding the mechanism of cortisol sensitivity in immune cells could lead to development of new treatment strategies. While some tissues benefit from glucocorticoid treatment, other tissues, might not. Future treatment strategies, aiming at timing and tissue specific regulation of glucocorticoids, might benefit patients with sepsis or other immune and inflammatory diseases.

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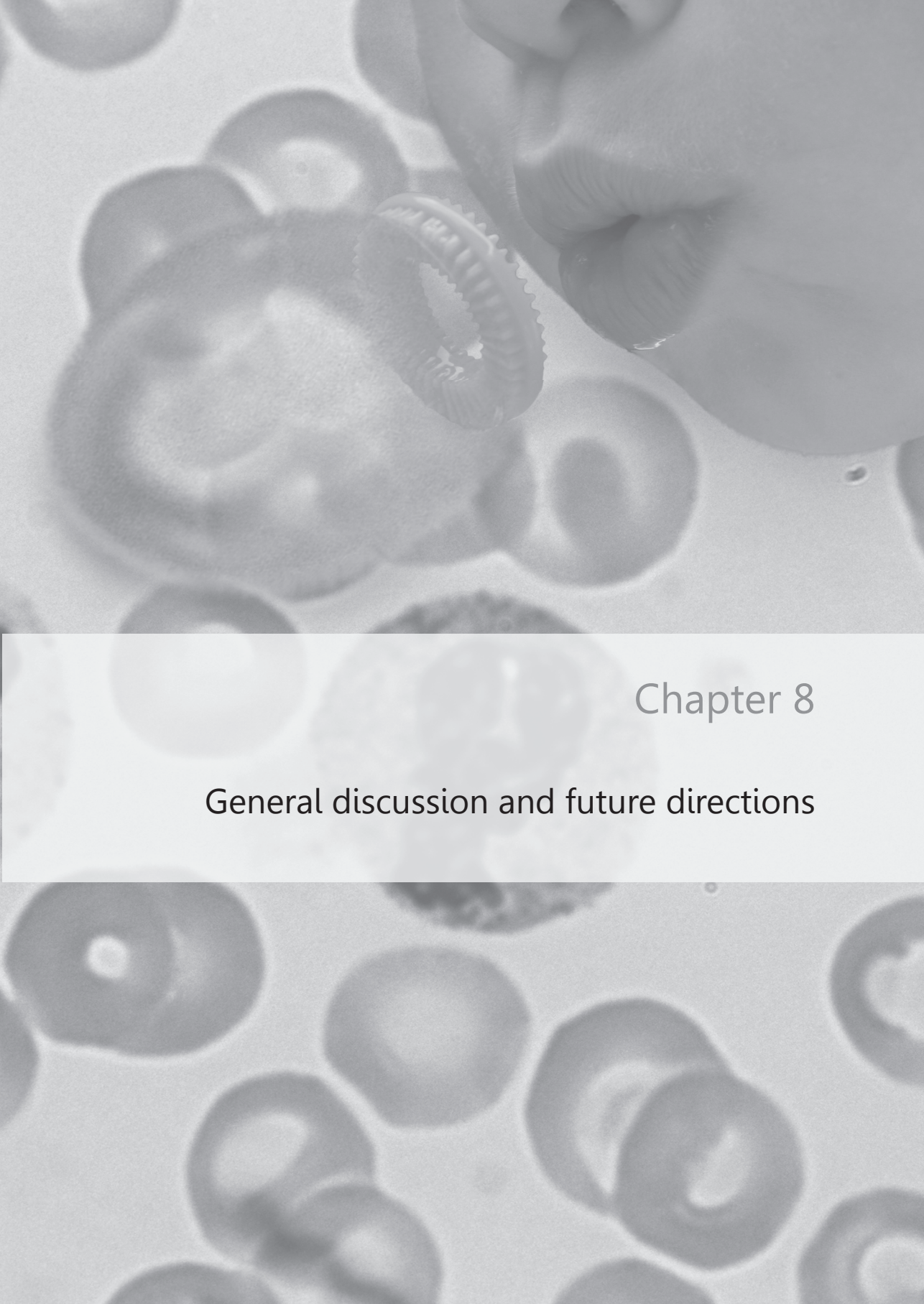

Chapter 8

General discussion and future directions

GENERAL DISCUSSION AND FUTURE DIRECTIONS

8.1 Rationale

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8.2 Implications and speculations

-

8.3 Limitations

-

8.4 Future directions

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References

8.1 RATIONALE

Individual variability in cortisol sensitivity has important implications for health profile and disease risk, the course of the disease, response to corticosteroid treatment, and development of novel treatment strategies. Insight in mechanisms determining glucocorticoid sensitivity could benefit future health care strategies like determination of risk profiles, individualization of treatment strategy and corticosteroid dosage.
The research described in this thesis aimed at studying genetic and post-genomic mechanisms that regulate cortisol sensitivity and to study its influence on the immune system and inflammation.

8.2 IMPLICATIONS AND SPECULATIONS

Glucocorticoid receptor haplotypes (Chapter 2+3).

Haplotypes from the 4 functional GR polymorphisms*: ER22/23EK*, *N363S*, *Bcl1* and *GR-9β* were constructed (Fig 1). All polymorphisms were mutually exclusive except *ER22/23EK*, which was always present in combination with *GR-9β*, but not the other way around (Fig 1). In this thesis, a focus was set on the haplotype characterized by the *GR-9β* polymorphism, because of its relationship with the immune system. This haplotype was found to be common in a large population based Caucasian cohort. The allele frequency was 14.5%; as a consequence, 25% of the population was heterozygous carrier and 2% was homozygous carrier of this haplotype.

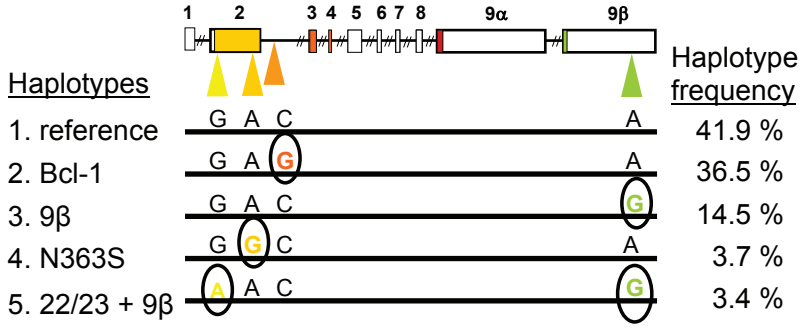


Figure 1. Schematic representation of the glucocorticoid receptor gene, variant haplotypes and allele frequencies.

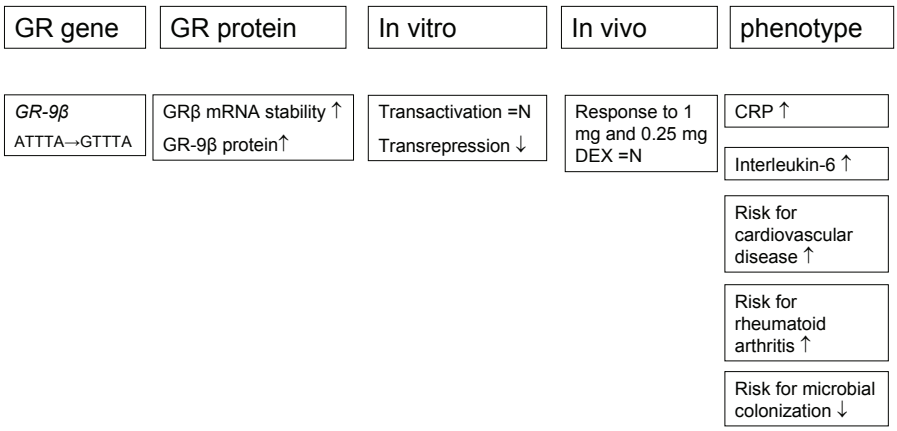


Figure 2. The glucocorticoid receptor gene variant GR-9β: from gene to phenotype.

Genetic variants: news on functionality and possible mechanisms.

New data on possible mechanisms by which the GR polymorphisms exert their effects have been published.

- *ER22/23EK*.

The *ER22/23EK* polymorphism affects GC sensitivity in a remarkable way. It has recently been shown that both AUG-1 and AUG-27 in the GR mRNA are used as translation start sites and that the GR protein resulting from translation starting at AUG-27 (termed GR-B) has greater transactivating activity than that starting from AUG-1 (termed GR-A) [1]. The *ER22/23EK* polymorphism induces changes in the secondary structure of the GR mRNA that lead to a shift in the balance between usage of AUG-1 and AUG-27 in favour of the "weaker" GR-A (starting at AUG-1) [2]. Transactivating capacity, in both transfection experiments and peripheral blood mononuclear lymphocytes of carriers of this polymorphism resulted in an increased transactivating capacity, both *in vitro* and *ex vivo*. Transrepression, measured with a bioassay, measuring corticosteroid induced repression of interleukin-2 gene expression, seems to be unchanged because the isoforms are equally potent at inhibiting the transactivating activity of NF-kB [3].

- *N363S*.

The molecular mechanism through which the *N363S* exerts its effects is unknown. Functional assays with reporter gene systems and homologous down-regulation revealed only minor differences between the wild-type GR and *N363S* GR in both transiently and stably expressing cell lines [3, 4]. However, examination of the two receptors by human gene microarray analysis revealed a unique gene expression profile for *N363S*. The *N363S* seems to regulate a novel set of genes with several of the regulated genes supporting a potential role for this GR polymorphism in human diseases [4].

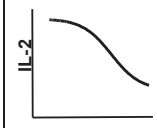
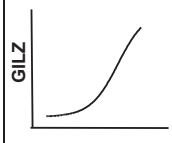
Mechanism		side-effects	treatment effect
Transrepression		HPA-axis suppression	Inflammation
		Infection susceptibility	
Transactivation		Cushing	
		Osteoporosis	
		Growth retardation	
		Diabetes mellitus	
		Glaucoma	

Fig 3. Corticosteroid treatment effects are mediated by glucocorticoid receptor transactivation and transrepression. Effects are classified by main mechanism.

- *GR-9*β

*GR-9*β is an A to G nucleotide substitution located in the 3' UTR of exon 9β, the terminal exon of the mRNA of the β isoform (nucleotide 3669 in X03348; rs 6198). The A to G nucleotide substitution is located in an 'ATTTA' motif (changing it to GTTTA). This 'ATTTA' motif is known to destabilize mRNA and decrease receptor protein expression in vitro [5]. The GRβ splice variant has been reported to have a dominant negative effect on GRα action [6, 7]. In vitro data show that the *GR-9*β polymorphism leads to a more stable GRβ mRNA and possibly to a relative GC resistance [8]. Ex vivo, GC-induced upregulation of GILZ mRNA via transactivation did not significantly differ in *GR-9*β homozygotes, while the downregulation of IL-2 expression via transrepression was decreased (Fig 2) [9].

GR-9β and clinical phenotype (Chapter 2-4).

The finding that persons, carrying the GR haplotype characterized by the *GR-9*β polymorphism, seem to have a decreased GC transrepression with normal transactivation could have important clinical implications. The side effects of corticosteroid treatment are thought to be mainly regulated by transactivation, while the immuno-suppressive effects are a result of GR transrepression (Fig3). Persons carrying the *GR-9*β haplotype revealed no significant differences in clinical measures of transactivating GR activity: Their body mass index, waist-hip-ratio, fat spectrum, insulin sensitivity and cortisol response to dexamethasone was not different compared to non-carriers (Chapter 2, Fig 2) [9]. However, clinical measures of transrepressive GR activity on the inflammatory and immune system (acquired and innate) were significantly associated with the *GR-9*β hap-

lotype. Less suppression of the immune system leads to a more active immune system. The more active immune system can be beneficial when needed for fighting infections (**I**). However a more active immune system can have an adverse effect on the risk of autoimmune disease (**II**). A chronically increased activity of the immune system could lead to a chronic pro-inflammatory state with eventually increased risk of atherosclerosis and cardiovascular disease (**III**) (Fig 2).

Ad I. In chapter 3, we describe a study on GR genetic variants and microbial colonization. The innate immune system is thought to play an important role in microbial colonization. In humans, the anterior nares are the primary ecological reservoir of Staphylococcus aureus (S.aureus). The ability of S. aureus to evade the inflammatory response of the host by surviving inside neutrophils has been shown to be a virulence factor. Impaired phagocytic activity is probably also a central factor in determining S. aureus nasal carriage [10, 11]. Persons homozygous of the GR-9β haplotype had a 68% reduced risk of persistent S. aureus nasal carriage (odds ratio 0.32; 95% confidence interval, 0.13-0.82). This is the first time that a human DNA polymorphism was associated with S. aureus nasal-carriage status, which is important for our knowledge on the mechanism of microbial colonization. Future studies could expand knowledge on the role of GR haplotypes on other microbial colonization like the methicillin-resistant Staphylococcus aureus (MRSA). In addition, it is presently unknown how persons, carrying the GR-9β haplotype, react to viral or bacterial infections, or how they respond to vaccination.

Ad II. Increased risk on auto-immune diseases like rheumatoid arthritis, in which regulation of the acquired immune system plays an important role, has been found in persons carrying the GR-9β polymorphism. A significant association with rheumatoid arthritis was found in a Dutch study[8] but not in a U.K.study [12]. Both these studies did not include haplotype analysis or the ER22/23EK polymorphism, which is linked to GR-9β. Recently preliminary data on GR haplotype association in a large study of patients with rheumatoid arthritis (n=368) compared to controls (n=5033) were presented. Carriers of the GR-9β haplotype (without ER22/23EK) had a higher risk of rheumatoid arthritis (odds ratio 1.26 (95%confidence interval 1.00-1.60) compared to controls (p=0.05) [13].

Ad III. In chapter 4 a study on GR genetic variants and inflammatory parameters, atherosclerosis and cardiovascular disease is described. The presence of a more active immune status in persons carrying the GR-9β haplotype is further supported by our finding of elevated levels of interleukin-6 (IL-6) and C-reactive protein levels measured with high sensitivity (hs-CRP) in a general elderly population[14]. The hs-CRP assay measures CRP levels in the reference range, and it should be noted that although the observed elevation is substantial and significant, it is within the normal range. Thus persons carrying the GR-9β haplotype seem to have a subtle but chronic pro-inflammatory status. In addition, persons homozygous for the GR-9β haplotype had an almost 3 times increased risk of cardiovascular disease (CVD). When studying gender-specific effects, in males

the risk of CVD increased to 3.5 times for homozygous GR-9β carriers, compared to the reference allele; however in women no significant difference was found (data not published). This could be a consequence of lack of power for the statistical analysis in this subgroup. In women, a total of 3006 was studied, 236 (8%) had a CVD event of which only 6 were homozygous GR-9β carriers. In men, a total of 1872 were studied, 257 (13%) had a CVD event, of which 13 were homozygous GR-9β carriers. It should be noted that in this group the incidence of CVD in women is lower compared to men. The effect of the GR polymorphism takes many years in the life-long process of atherosclerosis. Therefore the lack of association in women could also be a consequence of the GR-9β effect being overruled by the protective effect of estrogen during the pre-menopausal years.
Cortisol excess and stress are known risk factors for ischemic heart disease; however, the relationship with endogenous cortisol levels is less clear. Recent data on salivary cortisol, representing free cortisol, which is a more stable parameter in rapidly changing cortisol levels, was found to be associated with atherosclerosis [15]. The exact mechanism of cortisol influencing CVD risk is unknown. Our findings suggest that genetically determined cortisol sensitivity is involved in the pathogenesis of cardiovascular disease through inflammation. In the field of atherosclerosis and cardiovascular disease, this observation is important for understanding the mechanism and for identification of a subgroup at risk.
In summary, the GR-9β haplotype is associated with a more active immune system. Persons who are homozygous carriers of this haplotype might be protected for S.aureus colonization, while being predisposed to chronic autoimmune diseases, atherosclerosis and cardiovascular disease.

Characterization of cortisol sensitivity (Chapter 5)

Pathologic conditions

Cortisol resistance is a rare disease and diagnosis can be difficult. The main characteristics are hypercortisolism without signs of Cushing's syndrome, and hypertension, hypokalemia (mineralocorticoid excess) and with hirsutism, acne, male pattern baldness (androgen excess) [16, 17]. The clinical presentation in children can be a premature pubarche, increased growth velocity, advanced bone age as a result of increased androgens [16]. The 1 mg dexamethasone suppression test is used as a diagnostic tool (Fig 4). For children, the dexamethasone dose should be adjusted to their lower body surface compared to adults. Further characterization of the molecular mechanism can be performed in an experimental setting (Fig 4). For example, in a patient, who needed only low dose of corticosteroids for a postmortem donor kidney transplant, abnormal cortisol sensitivity was suspected. Expression of the GR splice variants was analyzed by quantification

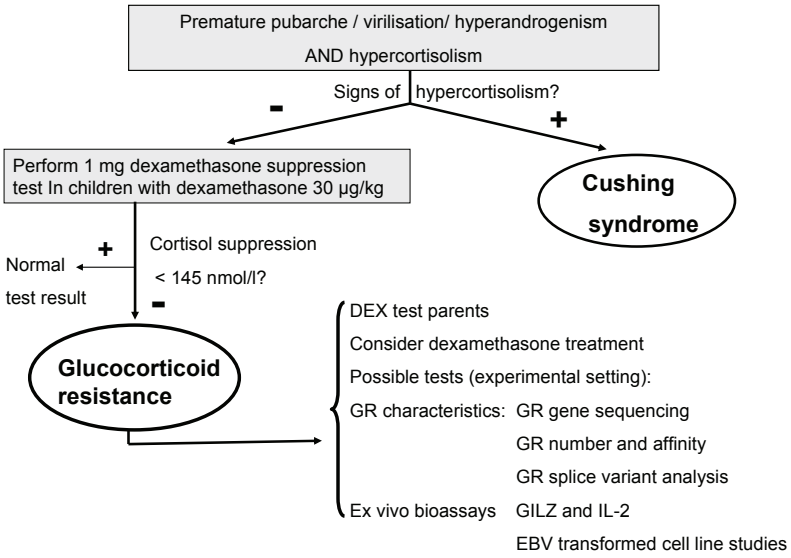


Figure 4. Diagnostic pathway for patients with suspected cortisol resistance

of mRNA copies of the GR using real-time Q-PCR, measured in peripheral blood mononuclear cells of patients and controls. He showed 3-4 fold higher GRβ expression levels, although no differences for expression of GRα and GR-P mRNA splice variants were found (Fig 5).

Cortisol hypersensitivity.

A patient with hypocortisolism and Cushing's syndrome-like manifestations has been described only once before [18]. We described a patient with cortisol hypersensitivity with increased glucocorticoid receptor number [19]. The patient was 13-yr-old, presenting with clinical features of Cushing's syndrome (progressive obesity, growth retardation with retarded bone age and osteoporosis). Fasting serum-and 24-hours urinary cortisol was below the reference range. For asthma, she used low-dose inhalation GCs (budesonide 200 µg/d). Ex vivo, 70% more GR mRNA expression was measured, corresponding with increased GR number measured in leukocytes in the ligand-binding assay. After stopping inhalation GCs, symptoms reversed partially. She was advised to consider adjusting corticosteroid dose whenever treatment is needed in the future.

The clinical conditions of cortisol resistance and hypersensitivity can be suspected from specific signs and symptoms described above. Recognition of this disease has important clinical implications for treatment. In addition, these 'experiments of nature' can be used to study mechanisms determining cortisol sensitivity.

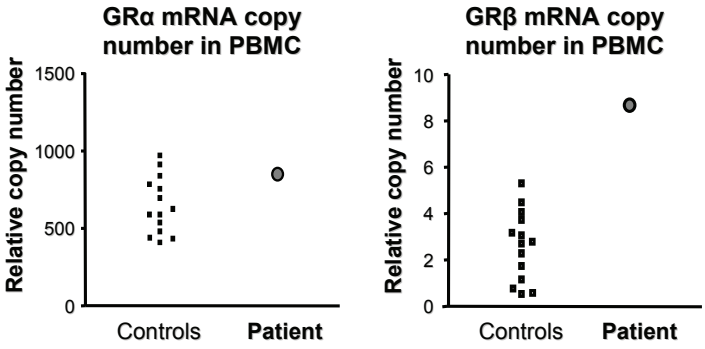


Figure 5. GRα mRNA and GRβ mRNA in a renal transplant patient still alive after 33 years, despite only low-dose immunosuppressive medication

Cortisol sensitivity in the general population.

It is important to realize that besides the extremes as seen in pathologic conditions, cortisol sensitivity has a wide variability in the general population showing a Gaussian distribution. This implicates that 2-10% (depending on percentile used) of the general population is either relative resistant or hypersensitive to cortisol.

Assessing glucocorticoid sensitivity

For characterization of GC sensitivity, measurement at different levels and in different assays is necessary. There is a variety of possible tests: cortisol rhythm, dexamethasone suppression tests (1 mg or 0.25 mg), GR gene polymorphisms, mutations, GR binding affinity, GR bioassay (GILZ en IL2), GR mRNA splice variants. Changes in GC sensitivity can be caused by decreased GR number, decreased GR DNA binding, GR thermolability, impaired translocation to the nucleus, altered interaction with co-activators, or changes in splice variant levels.

Sepsis

Pitfall in studying leukocytes of sepsis patients (Chapter 6)

Leukocytes play a critical role in sepsis. Studying the changing characteristics of leukocytes during sepsis is important for a better understanding of its pathophysiological sequelae. To date, the ficoll separation technique is often being used to study mononuclear cells in different clinical research settings. Our study showed that there is a pitfall in using this technique when studying sepsis patients. In these patients, the mononuclear cell fraction is highly contaminated with granulocytes (Fig 6). Additional separation tech-

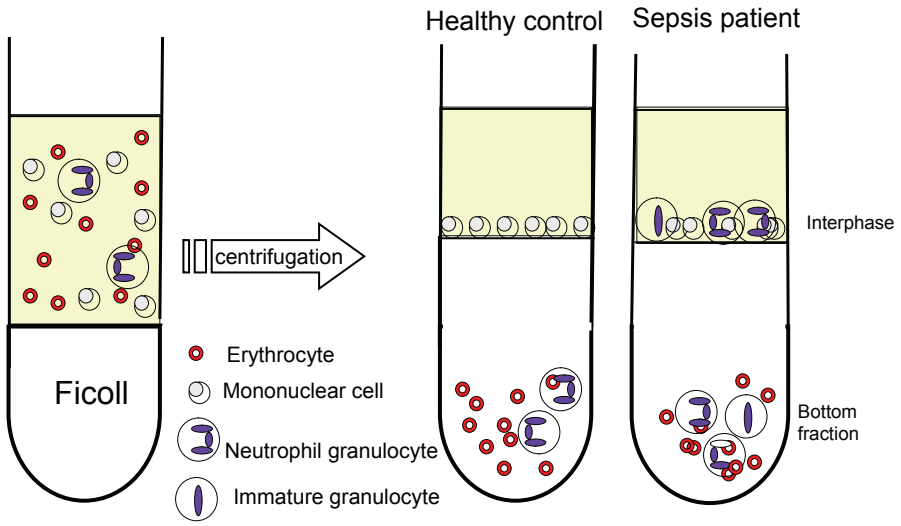


Figure 6. Ficol separation of leukocytes

niques are necessary to obtain a pure mononuclear cell fraction. In contrast to mononuclear cells, granulocytes can be separated with high purity.

Transient changes in cortisol sensitivity during sepsis (Chapter 7).

To study transient changes in cortisol sensitivity during sepsis, we measured GR splice variant expression in granulocytes of children with sepsis. Children with sepsis showed a transient depression of the glucocorticoid receptor splice variants GRα and GR-P mRNA in their neutrophils. This feature may represent a tissue specific adaptation during sepsis leading to increased cortisol resistance of neutrophils. Understanding tissue specific mechanisms of cortisol sensitivity could lead to development of new treatment strategies. While some tissues benefit from glucocorticoid treatment, other tissues, might not. Future treatment strategies, aiming at timing and tissue specific action of glucocorticoids, might benefit patients with sepsis or other immune and inflammatory diseases.
The GRα splice variant is seen as the most important for determining cortisol effect. Other splice variants like GR-P and GRβ also seem to play a role. We previously identified GR-P, which is present in amounts of about 20% of GRα and may increase the effect. Interestingly, in sepsis patients, we found a similar depression of GR-P mRNA expression compared to GRα. However, studies on GR-P are scarce; its role is still largely unclear and could be interesting for future studies. Much controversy exists on the GRβ splice variant[20, 21]. The expression of GRβ mRNA is very low, about 0,1% of GRα, although at a protein level it might be more prevalent. GRβ might be important in fine-tuning GR

effects. Several studies suggest a dominant negative effect [6] which leads to cortisol resistance and it has been associated with several auto-immune diseases like asthma, colitis ulcerosa and rheumatoid arthritis. GRβ expression in corticosteroid-insensitive asthma was particularly high in airway T cells, which are thought to play a major role in the pathogenesis of asthma [22]. Recently, in vitro studies showed GRβ upregulation upon pro-inflammatory cytokine stimulation leading to cortisol resistance of human airway smooth muscle cells [23]. In neutrophils, increased levels of GRalpha/GRbeta heterodimers were found in neutrophils as compared with mononuclear cells. Transfection of mouse neutrophils, which do not contain GRbeta, resulted in a significant reduction in the rate of cell death when treated with dexamethasone. High expression of GRbeta by human neutrophils may provide a mechanism by which these cells escape glucocorticoid-induced cell death. Moreover, upregulation of GRβ by proinflammatory cytokines such as IL-8 further enhanced their survival in the presence of glucocorticoids during inflammation [24]. During sepsis, we did not find a significant change of this splice variant in neutrophils. The expression of GRβ is variable in different tissues and therefore is an interesting splice variant from the perspective of tissue specific cortisol resistance. Future studies looking at GRβ expression in different tissues, at different developmental stages and in different diseases will elucidate its role further.

8.3 LIMITATIONS

• Although the genetic variants show associations with intermediate and endpoint parameters, the conclusions should be considered as hypothesis generating and replication studies are needed.

• Genetic variations in the general population are associated with differences in cortisol sensitivity within the normal range. Thus we should not overestimate their effects. One would expect the effects to be either subtle or obvious only after life-long exposure.

• Tools for individual measurement of cortisol sensitivity are still time consuming, usually a large volume of blood is needed, and these assays show a wide range of variation. Also, they are at present only possible in a research setting. Reliable, validated and fast diagnostic tools for measurement of cortisol sensitivity in adults and children are necessary.

• It should be taken into account that studying the effects of glucocorticoids is complicated by the intricate system of metabolism, feedback, genetic variants, post-genomic

splice variants, promoter variants, cofactors, posttranslational modification and tissue specific regulation.

8.4 FUTURE DIRECTIONS

- Genetic GR variants could be used in identifying individual genetic risk profiles for disease or their treatment.

- The study of GR gene variants is currently focused on several known polymorphisms, which are thought to be functional. Over the past decade, with the development of high throughput DNA sequencing protocols and advanced computational analysis methods, it has been possible to generate assemblies of sequence. In the future newly identified genetic variants based on SNP's or non-SNP genetic alterations, coming available from the human genome database, could be studied. By using novel haplotype assembly strategies, a definitive molecular portrait of a diploid human genome can be used and can lead to individualized genomic information.

- Genetic GR analysis should aim at expanding possibilities of studying the promoter region, co-factors and other tissue specific regulators of GR function like 11-β-HSD. *GR-9*β could be used as a risk marker for cardiovascular disease in intervention trials or in genetic risk profile screening studies. In these studies, *GR-9*β could be used as an additional risk marker to hs-CRP and cholesterol.

- The GR haplotype characterized by *GR-9*β is an interesting genotype to study in inflammatory and autoimmune diseases and in association with the success of GC treatments.
  The GR haplotype characterized by *GR-9*β and the identification of GR splice variant regulation could be an interesting model for development of immunosuppressive therapies with fewer side effects.

- Farmacogenetic development of improved GC treatment strategies can be seen from 2 perspectives:

- 1. from the patient perspective: adjusting the GC dose to individual GC sensitivity.
  2. from the drugs perspective: aiming at dissociated (more transrepression, less transactivation) and tissue specific effects.

- Development of diagnostic tools for the measurement of individual cortisol sensitivity is warranted for adults and children. The development of diagnostic tests should aim at easy, low cost, fast and especially reliable tests. These tests should preferentially use salivary cortisol over serum cortisol.

- Sepsis research on cortisol effects, needs to be studied at a tissue level in different tissues, at different time points, at different ages and in different diseases (like infectious diseases, different severity of disease or auto-immune/inflammatory diseases).

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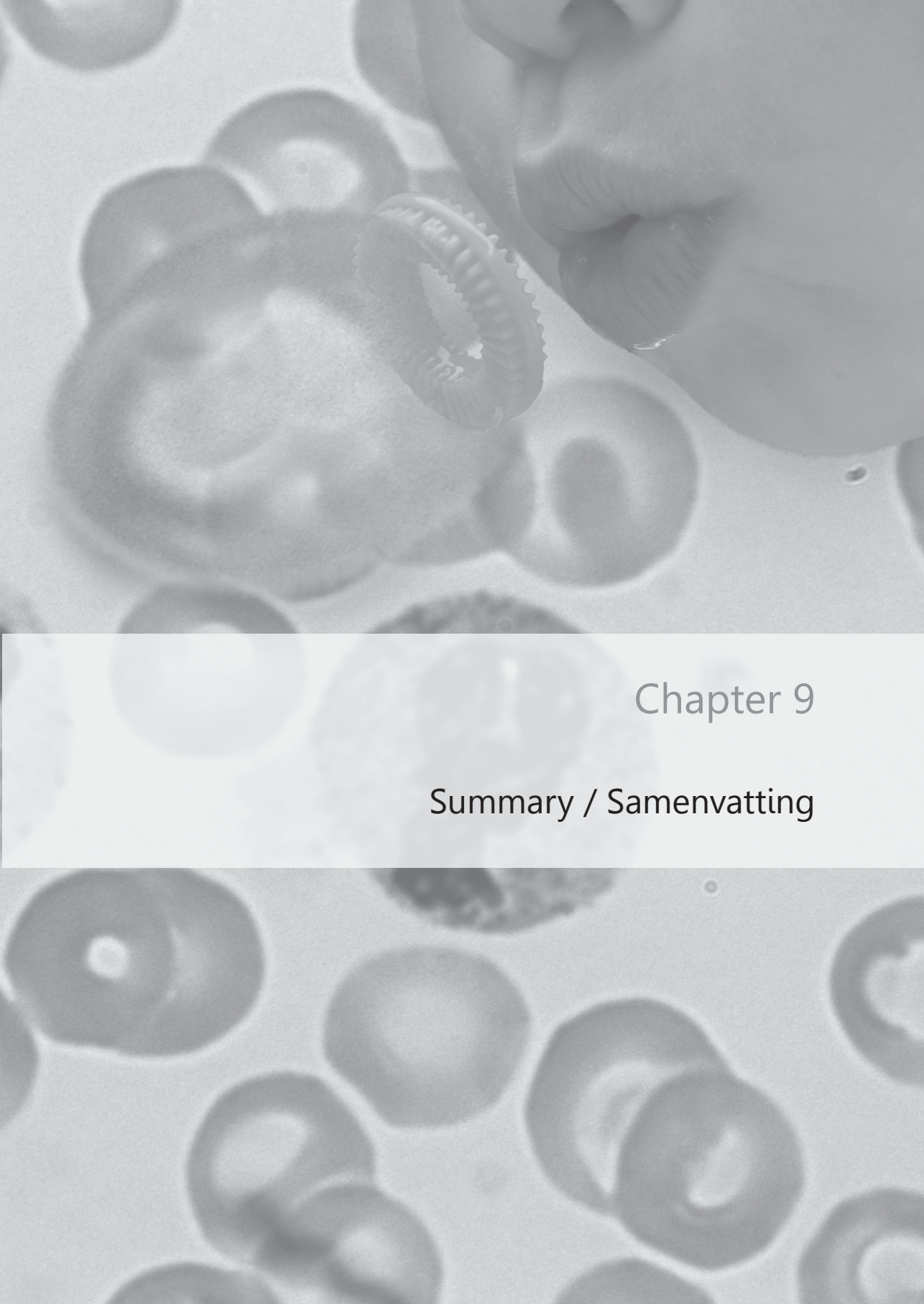

Chapter 9

Summary / Samenvatting

SUMMARY

This thesis describes glucocorticoid receptor effects on the immune system and inflammation. Results from population based studies, in vitro and ex vivo assays and patient studies are described.

A general introduction in glucocorticoid physiology, the molecular mechanisms of glucocorticoid receptor function and human (patho)physiology of cortisol sensitivity are reviewed in **chapter 1**. Special focus is put on the immune system and related diseases like sepsis. The aim of this thesis was to study molecular mechanisms of glucocorticoid receptor effects on cortisol sensitivity, with a focus on the immune system and inflammation.

The results described in **chapter 2** show persons carrying the glucocorticoid receptor gene variant (*GR-9β*) homozygous, seem to have a decreased glucocorticoid transrepression but not transactivation. Ex vivo, GC-induced upregulation of GILZ (glucocorticoid-induced leucine zipper) mRNA, via transactivation did not significantly differ in *GR-9β* homozygotes, whereas the down-regulation of IL-2 expression via transrepression was decreased. Data from a population based cohort study (n=216) were in line with these ex vivo findings. No association was found between the GR-9β gene variant and clinical or biochemical parameters that are mainly regulated by transactivation. In persons carrying *GR-9β* compared to non-carriers, no differences were found in body mass index, waist to hip ratio, fat spectrum, insulin sensitivity or cortisol response to dexamethasone. However, the trend toward higher CRP levels in homozygous *GR-9β* carriers might reflect their decreased transrepression.

The possible clinical implications of the decreased transrepression on the immune system in homozygous *GR-9β* carriers are described in **chapter 3**. Genotype-dependent variation in the sensitivity to glucocorticoids is associated with tolerance toward staphylococcal nasal colonization in a population-based study (n=2929). Persons carrying the glucocorticoid receptor gene variant *GR-9β* homozygous have a 68% reduced risk of persistent Staphylococcus aureus nasal carriage, whereas carriers of the 22/23EK gene variant displayed an 80% increased risk compared to non carriers.

The possible clinical implications of the decreased transrepression on inflammation in homozygous *GR-9β* carriers are described in **chapter 4**. In this chapter, a genotype-dependent variation in the sensitivity to glucocorticoids is associated with cardiovascular disease in a population-based study (n=7983). Persons carrying the glucocorticoid receptor gene variant *GR-9β* homozygous have a more than twice increased risk

of myocardial infarction and an almost 3 times increased risk of coronary heart disease, compared to non-homozygous persons. In addition, their levels of C-reactive protein, interleukin-6 and carotis intima media thickness were higher. These findings suggest that the *GR-9β* gene variant is related to a more active pro-inflammatory system.

Chapter 5 illustrates several strategies to define abnormalities in glucocorticoid sensitivity by describing nine patients with glucocorticoid sensitivity disorders. Differences in glucocorticoid receptor number per cell, glucocorticoid receptor affinity, glucocorticoid receptor splice variants and effects on transactivation or transrepression of the glucocorticoid sensitive genes glucocorticoid-induced leucine zipper (GILZ) and Interleukin-2 (IL-2) were observed between patients and healthy controls. Exploring glucocorticoid sensitivity disorders might give insight into the mechanistic background of glucocorticoid regulation in the human body.

The results described in **chapter 6** demonstrate that in sepsis patients, the ficol separation technique is not suitable for studying mononuclear cells, but can be used for studying granulocytes with high purity. In healthy controls, the interface of ficol-separated leukocytes consists mainly of mononuclear cells (88-100%). However, in sepsis patients, the interface is highly contaminated with granulocytes (48-89%). The bottom fraction, containing granulocytes, shows a high purity in sepsis patients and healthy controls (>92%). In future studies, additional separation techniques are warranted for isolation of mononuclear cells in sepsis patients. Cell content and purity should be checked.

In **Chapter 7**, 23 children with sepsis were studied. The glucocorticoid receptor mRNA levels in neutrophils were analysed longitudinally during illness and after recovery. The results show a transient depression of glucocorticoid receptor expression in neutrophils of children with sepsis. The mRNA levels of glucocorticoid receptor splice variants GR-α and GR-P were decreased on day 0 and showed a gradual recovery on days 3 and 7 and normalized after recovery. The mRNA levels of glucocorticoid receptor splice variant GR-β did not change significantly during sepsis. The changes in glucocorticoid receptor expression may represent a tissue specific adaptation during sepsis, leading to cortisol resistance of neutrophils. Future treatment strategies, aiming at timing and tissue specific regulation of glucocorticoids, might benefit patients with sepsis.

Chapter 8 contains a general discussion and future directions. The findings in this thesis are put into a broader perspective by discussing the rationale, implications, speculations, limitations and future directions.

SAMENVATTING

Dit proefschrift beschrijft glucocorticoid receptor effecten op het immuun systeem en inflammatie. De resultaten van populatie studies, in vitro en ex vivo assays en patiënten studies worden beschreven.

Hoofdstuk 1 is een algemene introductie in glucocorticoid fysiologie, moleculaire mechanismen en functie van de glucocorticoid receptor, en de (patho-) fysiologie van cortisol gevoeligheid in de mens. Hierbij wordt speciale aandacht besteed aan het immuun systeem en gerelateerde ziekten zoals sepsis. Het doel van dit proefschrift was het onderzoeken van de moleculaire mechanismen van glucocorticoid receptor effecten op cortisol gevoeligheid en het immuun systeem.

De resultaten beschreven in **hoofdstuk 2** laten zien dat personen die homozygoot drager zijn van de glucocorticoid receptor gen variant *GR-9β*, een verminderde glucocorticoid transrepressie hebben met normale transactivatie. In ex vivo assays is de transactivatie, gemeten via glucocorticoid geïnduceerde upregulatie van GILZ (glucocorticoid-geïnduceerde leucine zipper) mRNA expressie, niet significant anders in homozygote *GR-9β* dragers ten opzichte van niet-homozygoten. De transrepressie, gemeten via glucocorticoid geïnduceerde downregulatie van IL-2 (interleukin-2) mRNA expressie, is wel verminderd in homozygote *GR-9β* dragers. De bevindingen in een populatie studie (n=216), sluiten hier bij aan. In de klinische en biochemische parameters die voornamelijk via glucocorticoid transactivatie worden gereguleerd, werd geen associatie gevonden met de GR-9β gen variant. Geen associatie werd gevonden voor parameters als body mass index, buik:heup ratio, vet spectrum, insuline gevoeligheid of cortisol respons op dexamethason. De gevonden trend naar hogere serum C-reactive protein concentraties in homozygote *GR-9β* dragers zou het gevolg kunnen zijn van een minder onderdrukte inflammatie status ten gevolge van verminderde glucocorticoid transrepressie.

Hoofdstuk 3 beschrijft aspecten van de klinische implicaties op het immuun systeem van de verminderde glucocorticoid transrepressie in homozygote *GR-9β* dragers. Genotype afhankelijke variatie van het glucocorticoid receptor gen is geassocieerd met bacteriële kolonisatie in een populatie studie (n=2929). Personen, homozygoot drager van de glucocorticoid receptor gen variant *GR-9β*, hebben 68% reductie van de kans op persisterend Staphylococcus aureus dragerschap in de neus, terwijl dragers van de 22/23EK gen variant een 80% toename van risico hebben.

Hoofdstuk 4 beschrijft aspecten van de klinische implicaties op inflammatie van de verminderde glucocorticoid transrepressie in homozygoot *GR-9β* dragers. Genotypenafhankelijke variatie in gevoeligheid voor glucocorticoiden is geassocieerd met hart- en vaatziekten. In een

populatie onderzoek (n=7983) hebben personen, homozygoot drager van *GR-9β,* een 2x verhoogd risico op een hartinfarct en een 3x verhoogd risico op coronair hartlijden, vergeleken met niet homozygoten. Daarbij hebben ze ook hogere serum concentraties van C-reactive protein, interleukin-6 and carotis intima media dikte. Deze bevindingen suggereren dat de *GR-9β* gene variant gerelateerd is aan een actiever pro-inflammatoir systeem.

Hoofdstuk 5 illustreert strategieën om afwijkingen in glucocorticoid gevoeligheid te definieren. Bij patiënten (n=9) met een stoornis in glucocorticoid gevoeligheid werden verschillen gevonden in glucocorticoid receptor aantal per cel, glucocorticoid receptor affiniteit, glucocorticoid receptor isoformen en effecten op transactivatie en transrepressie van glucocorticoid gevoelige genen (GILZ en IL-2) ten opzichte van gezonde controles. Het onderzoeken van stoornissen in glucocorticoid gevoeligheid kan inzicht geven in de mechanismen van glucocorticoid regulatie in het menselijk lichaam.

De resultaten beschreven in **hoofdstuk 6** laten zien dat de ficol scheidingstechniek niet geschikt is voor het bestuderen van mononucleaire cellen in sepsis patiënten. Deze techniek kan wel gebruikt worden voor het bestuderen van granulocyten. In gezonde controles bestaat na scheiding van witte bloedcellen de interfase voornamelijk uit mononucleaire cellen (88-100%). Daarentegen is de interfase van sepsis patiënten sterk verontreinigd met granulocyten (48-89%). De bodem fractie bestaat bijna zuiver uit granulocyten in zowel gezonde controles als in sepsis patiënten (>92%). Toekomstige studies zullen aanvullende scheidingstechnieken moeten gebruiken voor de isolatie van zuivere populaties mononucleaire cellen in sepsis patiënten. Het nodig om cel samenstelling en zuiverheid te controleren.

In **hoofdstuk 7**, worden 23 kinderen met sepsis bestudeerd. De glucocorticoid receptor mRNA expressie in neutrofiele granulocyten werd longitudinaal geanalyseerd tijdens ziekte en na herstel. De resultaten laten een transiënte onderdrukking van glucocorticoid receptor expressie zien in neutrofiele granulocyten van kinderen met sepsis. De mRNA expressie van glucocorticoid receptor isoform GR-α and GR-P zijn onderdrukt op dag 0 en tonen een geleidelijk herstel op dag 3 and 7 en normaliseren na herstel. De mRNA expressie van glucocorticoid receptor isoform GR-β veranderde niet significant tijdens sepsis. De veranderingen in glucocorticoid receptor expressie tijdens sepsis zou een weefsel specifieke adaptatie kunnen zijn, die tijdens sepsis de neutrofiele granulocyten resistenter maakt voor cortisol. Toekomstige behandelstrategieën gericht op de juiste timing van toediening en gericht op weefsel specifieke regulatie van glucocorticoiden zouden de patiënt ten goede kunnen komen.
Hoofdstuk 8 bevat een algemene discussie van de resultaten met suggesties voor de toekomst. De bevindingen in dit proefschrift worden in een breder perspectief gezet door het bediscussiëren van de rationale, implicaties, speculaties, beperkingen en suggesties voor de toekomst.

DANKWOORD

Graag wil ik een woord van dank richten tot alle mensen die aan het onderzoek, beschreven in dit proefschrift, hebben bijgedragen. Het is een voorrecht om met zoveel getalenteerde en bijzondere mensen samen te werken.

Beste ouders, kinderen, patiënten, hun behandelaars en anonieme controle personen, dank voor jullie belangeloze bereidheid tot medewerking aan het onderzoek.

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CURRICULUM VITAE

1967	Geboren te Wormerveer
1979- 1985	Gymnasium, Rijnlands Lyceum te Wassenaar
1985- 1990	Doctoraalfase geneeskunde Rijks Universiteit Leiden
1987	Summerschool Harvard University, Cambridge, U.S.A.
1990	Klinische stage inwendige geneeskunde, Royal Brisol Infirmary, Bristol, U.K
1991	Klinische stage chirurgie, St Elisabeth Hospitaal, Curaçao
1992	Artsexamen Rijks Universiteit Leiden
1992-1998	Opleiding tot kinderarts, Universitair Medisch Centrum Groningen, opleiders Prof. dr H.S.A. Heymans en W. Fetter. Onderzoek naar een nieuwe mutatie in het vasopressine gen in een familie met diabetes insipidus. Isala Klinieken te Zwolle, afdeling kindergeneeskunde, i.s.m. klinische chemie, inwendige geneeskunde en Rudolf Magnus Instituut voor hersenwetenschappen, te Utrecht.
1998-2002	Subspecialistische opleiding tot endocrinoloog, Erasmus MC-Sophia, o.l.v. Prof S.L.S. Drop Onderzoek naar in vitro expressie van CYP 17 mutanten, Laboratorium inwendige geneeskunde, o.l.v. Prof. dr. F.H. de Jong Onderzoek naar effect van topiramaat bij kinderen met morbide obesitas, afdeling kindergeneeskunde ism neurologie, Erasmus MC

2002-2007	Projectleider van onderzoek naar de effecten van een multidisciplinair gedragstherapeutisch behandelprogramma voor kinderen met overgewicht of obesitas. Productie werkboeken, opzetten training en uitrol van het behandelprogramma in Nederland. St Franciscus Gasthuis afdeling kindergeneeskunde i.s.m. klinische psychologie, diëtetiek, fysiotherapie en Medische psychologie & Psychotherapie Erasmus MC
	Werkzaam als kinderarts, St Franciscus Gasthuis te Rotterdam
2003-2005	Projectleider onderzoek naar de prevalentie van vitamine D deficientie bij pasgeborenen van gesluierde of donker gepigmenteerde moeders. St Franciscus Gasthuis te Rotterdam, afdeling kindergeneeskunde i.s.m. afdelingen klinische chemie en Gynaecologie
2002-2007	Promotie-onderzoek: "Glucocorticoid receptor effecten op het immuun systeem en inflammatie."
2005- heden	Staflid kindergeneeskunde, subafdeling endocrinologie, Erasmus MC- Sophia
Erkenningen	2001 Jonge auteursprijs, Nederlands Tijdschrift voor Geneeskunde 2007 Posterprijs, Congress European Society Pediatric Endocrinology
Persoonlijk	Getrouwd met Andrew C. Kruseman Aretz 4 kinderen: Pepijn (1998), Fin (1999), Tisse (2001), Victoria (2005) Hobbies: Hardlopen, schaatsen, roeien.

LIST OF PUBLICATIONS

1. van den Akker ELT, Koper JW, van Rossum EFC, Dekker JMHJ, Russcher H, de Jong FH, Uitterlinden AG, Hofman A, Pols HA, Witteman JCM, Lamberts SWJ (2008) **Glucocorticoid receptor gene and risk of cardiovascular disease**. Arch Intern Med 168:1-7

2. van den Akker ELT, Koper JW, Joosten K, de Jong FH, Hazelzet JA, Lamberts SWJ, Hokken-Koelega ACS (2008) **Glucocorticoid receptor expression is decreased in neutrophils of children with sepsis**. Submitted

3. van den Akker ELT, Baan CC, van den Berg B, Russcher H, Joosten K, Hokken-Koelega ACS, Lamberts SWJ, Koper JW (2008) **Ficoll separated mononuclear cells from sepsis patients are contaminated with granulocytes**. Int Care Med in press

4. Voorhoeve PG, van den Akker ELT, van Rossum EFC, Koper JW, Kemper HGG, Lamberts SWJ, Delemarre-van de Waal HA (2008) **Glucocorticoid receptor Bcl1 variant is associated with increased body fatness in youngsters. Differences between generations.** Submitted

5. van den Akker ELT, Voorhoeve PG, Kemper HGG, Lamberts SWJ, Delemarre-van de Waal HA, Hokken-Koelega ACS (2008) **Glucocorticoid receptor gene polymorphism more frequent in children born small for gestational age without catch-up growth**. Submitted

6. van den Akker ELT, Puiman PJ, Groen M, Timman R, Jongejan MT, Trijsburg W (2007) **A cognitive behavioral therapy program for overweight children**. J Pediatr 151:280-283

7. Dijkstra SH, van Beek A, Janssen JW, de Vleeschouwer LH, Huysman WA, van den Akker ELT (2007) **High prevalence of vitamin D deficiency in newborn infants of high-risk mothers.** Arch Dis Child 92:750-753

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10. Russcher H, Smit P, van Rossum EF, van den Akker ELT, Brinkmann AO, de Heide LJ, de Jong FH, Koper JW, Lamberts SW (2006) **Strategies for the characterization of disorders in cortisol sensitivity**. J Clin Endocrinol Metab 91:694-701

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12. Aarsen FK, van den Akker ELT, Drop SL, Catsman-Berrevoets CE (2006) **Effect of topiramate on cognition in obese children**. Neurology 67:1307-1308

13. Russcher H, Smit P, van den Akker ELT, van Rossum EF, Brinkmann AO, de Jong FH, Lamberts SW, Koper JW (2005) **Two polymorphisms in the glucocorticoid receptor gene directly affect glucocorticoid-regulated gene expression.** J Clin Endocrinol Metab 90:5804-5810

14. van den Akker ELT, Groen M., van 't Spijker A, Pot DJ, Trijsburg W (2005) **Favourable short-term effects of a multidisciplinary, behavioural therapy, group treatment for overweight or obese children.** Ned Tijdschr Geneeskd 149:1102-1106

15. Dijkstra SH, Arpaci G, Huijsman WA, Boot AM, van den Akker ELT (2005) **Seizures in foreign newborns due to maternal vitamin-D deficiency.** Ned Tijdschr Geneeskd 149:257-260

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20. Abbes AP, Bruggeman B, van Den Akker ELT, de Groot MR, Franken AA, Drexhage VR, Engel H (2000) **Identification of two distinct mutations at the same nucleotide position, concomitantly with a novel polymorphism in the vasopressin-neurophysin II gene (AVP-NP II) in two dutch families with familial neurohypophyseal diabetes insipidus**. Clin Chem 46:1699-1702